BRITISH JOURNAL OF CANCER

VOL. VI SEPTEMBER, 1952 NO. 3

"ULCER-CANCER" OF THE STOMACH.

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Received for publication June 3, 1952.

Does Chronic Gastric Ulcer tend to undergo Malignant Change?

The importance of this question is beyond doubt, both because of the high incidence of gastric ulcer, and because many patients suffering from it are still treated medically over long periods before reference to a surgeon. The answer to it is also of considerable importance to the surgeon, owing to the great difficulty of assessing at laparotomy whether early malignant change may have already supervened on chronic ulceration. If the development of secondary carcinoma is really as common as some pathologists' figures would appear to indicate, there might be a strong case for an even more radical gastrectomy than is usually carried out for such lesions.

Conflicting clinical and pathological evidence.

The consensus of opinion in the medical literature is that gastric ulcer is a definite predisposing cause of carcinoma, but there is profound disagreement among pathologists about the percentage of cases which undergo such malignant change. Udaondo (1939), in an extensive survey of the world literature, found estimates of the incidence of "ulcer-cancer" which ranged from 0 to 100 per cent, with most intermediate values represented. Obviously, some statistical uncertainty must be present in the majority of these assessments, yet there is usually quite inadequate evidence in the published data to lead to its detection. The only general conclusion that can be drawn from a study of these papers is that the authors' confidence in the more extraordinary estimates seems to vary inversely with the amount of evidence at their disposal.

Since, according to the Registrar-General's returns, something like 14,000 people die each year from cancer of the stomach, it is not surprising that the heavy incidence of gastric ulcer in the population should have suggested that there was some connection between the two conditions. More especially so, since a significant relationship has been traced between chronic ulcerative lesions elsewhere in the body and the subsequent development of malignancy. Yet clinical, as opposed to histopathological, evidence casts doubt on this relationship

as regards the stomach, and suggests that the incidence of malignant change must be very considerably lower than is suggested by most pathologists' figures.

Livingstone and Pack (1939) assess the average survival of patients admitted to hospital with inoperable cancer of the stomach at under 4 months, whilst the total duration of untreated primary gastric carcinoma is often under a year, from the onset of the first clinical symptoms until death supervenes. Chronic gastric ulcer, on the other hand, appears to be relatively benign. A high proportion of ulcers heal under medical treatment, the failure rate being quoted as 19·3 per cent by Marshall (1944) from the Lahey Clinic figures, and at less than 11 per cent in the series of 1500 cases treated by Brown and his colleagues (Brown, 1945). Analysis of the published data by Ivy, Grossman and Bachrach (1950) shows that the average time taken for the crater to disappear radiologically in these cases is less than 2 months; yet medically resistant ulcers may persist for many years without cancer supervening. Eusterman and Balfour (1936) state that the average duration of such lesions prior to operation is 7 years; whilst Ivy, Grossman and Bachrach (1950) conclude from a detailed study of the post-mortem incidence of gastric ulcer that it is "a disease of potentially great chronicity". Of these chronic cases only a relatively small number are eventually associated with the secondary development of malignancy—6·5 per cent in Hurst and Stewart's (1929) series. Furthermore, the favoured sites for the development of carcinoma and ulcer are predominantly different. In over 11,500 cases of gastric cancer quoted by Ewing (1940), approximately 60 per cent occurred in the prepyloric region; yet the figures quoted by Martin (1909), Hauser (1926), Ihre and Müller (1943), and Hurst and Stewart (1929) show that 60 per cent to 80 per cent of all gastric ulcer occurs along the lesser curvature. Obviously, ulcers do not degenerate over-willingly and "ulcer-cancer" must form a relatively rare complication.

All experienced pathologists recognise this fact—that the "ulcer-cancer" association represents only a fraction of the total incidence of carcinoma—and are generally very careful to underline the necessity for strict criteria of what constitutes microscopical evidence of malignant change in an antecedent ulcer. In this country these criteria have become increasingly restrictive, and the more authoritative workers such as Stewart (1926) are reluctant to make such a diagnosis unless the most rigorous conditions are satisfied. The importance of this is amply justified in view of the gravity of the problem at issue; particularly so since the ultimate verdict of malignancy must so frequently depend on a microscopical opinion. But, apart from the fact that no two histologists will wholly agree on the evidence which justifies such an opinion, the same pathologist not infrequently changes his criteria on reviewing the same series of specimens after a lapse of years. This, of course, is bound to occur as the result of increasing experience; yet it means that the findings of an individual pathologist over a lengthy series of cases are only likely to be sufficiently homogeneous for statistical purposes if the time involved in the assessment of his data is not too lengthy, in terms of his tendency to change his pathological criteria. Equally, it means that there is grave statistical objection to "averaging" blindly the data of different pathologists. Until pathologists in general are able to come to an agreement to assess their findings on common criteria as to what constitutes malignant change in an ulcer and what differentiates this from a cancer arising *de novo*, each set of figures must be judged independently on internal statistical criteria.

The danger of analogy.

There would, despite the apparently divergent clinical evidence, be no question of the entity of " ulcer-cancer " if all pathologists were fully agreed that it constituted a distinct, definable and recognised microscopical condition; that it possessed characteristics quite different from those of simple ulcer which might be associated in a non-causative sense with cancer; and that it was readily distinguishable from the regenerative processes frequently visible in chronic ulcerative lesions. The only statistical task then remaining would be that of estimating the frequency of its occurrence. But no such agreement on the microscopical findings and their histological interpretation appears probable as yet—even amongst the most experienced workers. On the contrary, the conflicting evidence and extreme variations in the published pathological data make any attempt at mathematical analysis of them futile.

As has already been suggested, the clinical evidence of malignant change in pre-existing gastric ulcer is a relative rarity in the collective experience of both surgeons and pathologists. Thus, as soon as doubts are cast on the infallibility of histological diagnosis, it would seem that the only logical basis for continued belief in " ulcer-cancer " as a comparatively common entity is the analogy of the causative influence of chronic inflammation on subsequent development of carcinoma elsewhere in the body. Such arguments by analogy may, however, be most fallacious, and can directly contravene the famous principle first enunciated by William of Occam several centuries ago—" *Entia non sunt multiplicanda praeter necessitatem.*" The importance of this principle in scientific thought, of which it forms one of the main pillars, arises from the fact that it is impossible in practice to prove a universal negative by inductive processes. An example will make this clear.

Our ancestors, influenced by superstition rather than by scientific method, were constantly inventing *ad hoc* explanations for things in terms of witches, goblins, evil eyes, fairies and leprechauns which the whole weight of philosophy and Christianity has not yet succeeded in completely eradicating. The survival of such hypotheses is understandable on the grounds that they are easy to conceive, better than no explanation at all and, once accepted, virtually impossible to disprove. No amount of vain searching for leprechauns proves that they do not exist. The most that can be said is that they are either few in number or very shy. And then we shall always be countered by the fellow who is positive he saw several the other night! Such hypotheses are by their very nature invulnerable.

In contrast, the opposite scientific hypothesis that the *genus* Leprechaun does not exist, except possibly as a freak of natural development in isolated instances, is extremely vulnerable. Indisputable evidence of widespread leprechaun activity will entirely refute it, even if no actual leprechaun can be produced for examination. It is essential that this characteristic be present in any scientific hypothesis, so that the collection of sufficient opposition evidence can establish its falsity.

Adoption of the null hypothesis.

Applying these basic principles to the problem of " ulcer-cancer," can it be said that this conception has really been adequately demonstrated clinically,

or even that it forms an essential entity in morbid anatomy? Or have we perhaps accepted its existence with little more justification than our ancestors had in welcoming the leprechaun? The very phraseology of the relevant literature—" the incidence of ulcer-cancer probably does not exceed x per cent "—suggests that this may be the case, and that pathologists are themselves engaged in an attempt to depreciate its frequency of occurrence. It would seem, therefore, that a more profitable approach to the problem will be to attack it from the opposite direction, by the assumption of a " null hypothesis". In other words, to investigate the situation on the postulate that ulcer and cancer always occur independently in the stomach, in the aetiological sense, and that their occasional intimate association is entirely a matter of pure chance.

Failing the demonstration of " ulcer-cancer " as a necessary and unmistakable microscopical entity, such a null hypothesis could still be challenged by statistical evidence that the relative frequency of occurrence of associated ulcer and cancer is significantly greater than the hypothesis predicts. If the frequency of such associated lesions is so high that it is unlikely to have arisen by chance, the aetiological connection would be established, but even then this might only indicate that ulcer is a favoured site for the development of a carcinoma destined to arise in any case. However, since it is on the relative frequencies of these lesions that the null hypothesis must stand or fall, it is now important to survey the position statistically. And this is best carried out in general terms in order to avoid errors present in the conflicting pathological figures, and any profitless mathematical resolution of them.

In such an investigation, general figures of cancer incidence are obviously valueless. The only data worthy of consideration are those which have been verified pathologically, and the analysis must therefore be carried from the population at large up to the point where gastric specimens arrive at the pathologist. In the statistical model of the situation about to be constructed, it is assumed that the specimens eventually arriving at the pathologist are drawn from a population whose characteristics with respect to both gastric ulcer and carcinoma may be regarded as stable over a period of time covered by any particular analysis; and that, by virtue both of its size and its autogenerative properties, the composition of this population remains substantially unaffected by the withdrawal of the specimens. It will also be assumed that ulcer and cancer occur quite independently.

Development of the statistical model.

In the population postulated there will be a fraction, **U**, of individuals with gastric ulcer and a fraction, **C**, with carcinoma of the stomach. The fractions without these lesions will therefore be $(1 - \mathbf{U})$ and $(1 - \mathbf{C})$ respectively, and the whole population will be assignable to four categories in accordance with the following schematic:

Category.	Characteristics.	Fraction of total population.
1	Having cancer but not ulcer	$\mathbf{C}(1 - \mathbf{U})$
2	Having ulcer but not cancer	$\mathbf{U}(1 - \mathbf{C})$
3	Having both ulcer and cancer	$\mathbf{UC}$
4	Having neither lesion	$(1 - \mathbf{U})(1 - \mathbf{C})$

If it could be shown that the ratio $\frac{\mathbf{UC}}{\mathbf{U}}$ (which measures the incidence of cancer among those in the population who have ulcer) was significantly greater than the quantity **C**; or that the ratio $\frac{\mathbf{UC}}{\mathbf{C}}$ (which measures the incidence of ulcer among those in the population who have cancer) was significantly greater than the quantity **U**; then this might be evidence that gastric ulcer is a predisposing cause of cancer. But there is no known reliable estimate of the value of **U**; whilst the value of **C**, though an approximate estimate of it might be obtained from the Registrar-General's returns, cannot be meaningly correlated with the particular sample of the population on which any particular series of pathological figures is based. These two ratios are the ones commonly quoted in the literature. Unfortunately, however, they are of little meaning, since stomachs resected for carcinoma are anything but an unbiased sample of all cases of gastric cancer, and the same is equally true in the case of gastric ulcer. Evidently something better than this simple approach is essential.

Let us therefore consider a model stomach, which for simplicity consists of only two compartments. It may be illustrated diagrammatically as follows:

Pyloric Zone	Lesser Curvature

The model has been deliberately restricted to these two regions as they constitute the main sites of origin of both ulcer and cancer, though there is no reason why a similar analysis should not be made, if desirable, using a model with more compartments. All lesions outside these chosen areas will be excluded from our following analysis. In the population at large there will be a fraction, u, of all ulcers which occur in the pyloric zone, and another fraction, c, of all cancers located in the same compartment of the model. The population at large will then fall into the categories illustrated in Fig. 1 with the frequencies stated, the left-hand compartments of the diagrams representing the pyloric zone and the right-hand ones the lesser curvature; the two lower sections showing the position with respect to the relative frequencies of double lesions occurring in the same region, compared with double lesions which are sited in separate regions of the stomach. We shall refer to such double lesions occurring in the same region of the stomach as " amalgamated lesions " throughout the remainder of this paper, even if they are actually separate. Within this limited selection of the whole data evidence might be found of a tendency for co-existing ulcer and cancer lesions to become amalgamated in this sense more frequently than predicted by the null hypothesis, on which basis the expected value of the ratio of amalgamated lesions to non-amalgamated lesions is seen from Fig. 1 to be—

$$A_1 = \frac{uc\mathbf{UC} + (1-u)(1-c)\,\mathbf{UC}}{u(1-c)\,\mathbf{UC} + c(1-u)\,\mathbf{UC}},$$

which, on cancelling throughout by **UC**, becomes—

$$A_1 = \frac{uc + (1-u)(1-c)}{u(1-c) + c(1-u)}.$$

If, in practice, the observed value for this ratio were significantly greater than this expected value, it would indicate that ulcer was at least a favoured site for

the development of a cancer, if not an actual aetiological factor in its development. It will be seen that this ratio has the statistical advantage that it does not include the imponderable quantities **U** and **C**, but depends only on the site distribution factors u and c. It will be convenient in future to refer to this ratio A_1 as the *Amalgamation Ratio*.

		$(1 - \mathbf{U})\,(1 - \mathbf{C})$
Ulcer		$u\mathbf{U}(1 - \mathbf{C})$
	Ulcer	$(1 - u)\,\mathbf{U}(1 - \mathbf{C})$
Cancer		$c(1 - \mathbf{U})\,\mathbf{C}$
	Cancer	$(1 - c)\,(1 - \mathbf{U})\,\mathbf{C}$
Ulcer	Cancer	$u(1 - c)\,\mathbf{UC}$
Cancer	Ulcer	$c(1 - u)\,\mathbf{UC}$
Ulcer and Cancer		$uc\mathbf{UC}$
	Ulcer and Cancer	$(1 - u)\,(1 - c)\,\mathbf{UC}$

FIG. 1.

A further interesting ratio emerges from the study of Fig. 1, which has the same desirable characteristics of independence of **U** and **C**. This is the ratio

of amalgamated lesions at the pylorus to amalgamated lesions at the lesser curve. The value for this ratio on the null hypothesis is—

$$D_1 = \frac{ucUC}{(1-u)(1-c)\overline{UC}},$$

which reduces on cancelling by **UC** to—

$$D_1 = \frac{uc}{(1-u)(1-c)}.$$

It will be convenient to refer to this ratio as the *amalgamation distribution ratio.* Whereas the *amalgamation ratio* simply indicates whether the total amount of amalgamation is greater than the expected value on the null hypothesis, this second ratio shows whether amalgamation occurs preferentially in one or other of the regions.

Effect of the selection chain.

The model shown in Fig. 1 gives the expected distribution of all cases of ulcer and carcinoma into the various categories. If the null hypothesis is true, we should expect that a random collection of pathological specimens from the population at large would agree with the predicted ratios within the limits of sampling error. But, of course, pathological specimens are not collected at random, and it is therefore essential to consider in some detail the effect of the selection chain along which patients have to pass before their stomachs become pathological specimens.

The first group illustrated in the model shown in Fig. 1, those cases which have neither ulcer nor cancer, will, of course, be entirely missing from the pathologist's data.

Consider next the two categories of simple ulcer. Is it likely that ulcer of the pyloric canal will have the same probability of passing along the reference chain to the pathologist as ulcer at the lesser curve? Prepyloric ulcer, in its uncomplicated form, is notoriously difficult to demonstrate radiologically but, once diagnosed with certainty, is usually operated on without delay, since so many of these lesions prove eventually to have been malignant since their onset. Benign ulcers at this site tend to give rise to early symptoms from the secondary pylorospasm which they cause; yet they are often undetected until pyloric obstruction supervenes later, as the result of gradual stenosis, or unless acute perforation occurs. On the other hand, lesser curvature ulcer does not tend to produce such early symptoms, but is easier to display radiologically. Perforation is rarer, but haemorrhage more liable to occur at a relatively early stage of its development, thus aiding clinical diagnosis. Yet because they often heal under medical régime, there is frequently a significant time-lag before such lesions reach the surgeon, during which death may intervene from repeated haematemeses.

Consider now the two similar cancer categories. Pyloric carcinoma is probably diagnosed earlier, on the whole, than is cancer of the body of the stomach, owing to the relatively rapid development of obstructive symptoms. On the other

hand, it is more often inoperable at laparotomy, and therefore is less likely to reach the pathologist as a specimen.

There is thus a complex selection mechanism operating along the whole of the reference chain, from the differential symptomatology in the patient through the medical practitioner or consulting physician to the surgeon. Only after all this does the stomach become a potential pathological specimen, the likelihood of which is again governed by the surgeon's technical skill, and the influence of this on his decision whether to operate and finally to resect. Moreover, still other factors may influence this selection of specimens, which will be different for each pathologist. The number of patients with gastric ulcer under observation by the medical side of the hospital will be one of these, for associated " ulcer-cancer " lesions will have a better chance of being diagnosed early if the chronic ulcer lesions are kept under continual scrutiny by the consultant physician rather than being referred back to their own family doctors for routine treatment. Further, the alteration in symptomatology resulting from the development of a secondary cancer in these cases is often relatively small in the early stages. Much depends on the vigilance and diagnostic skill of the physician concerned.

The almost universal tendency among reporters is to ignore these very important weightings of their figures, and to hope that in the end they will cancel each other out over a large series. Comparison of one series with another—or even the first and second part of the same observer's series—soon demonstrates that this does not happen. The fact is that each pathologist's series must be considered a unique set, not only because of his particular pathological criteria for " ulcer-cancer " but, perhaps even more, because his experience will include a unique set of weights in the data.

Introduction of the weighting factors.

It is now evident that the simple model shown in Fig. 1, which portrays the situation in the population at large, cannot be taken as an adequate picture of the expected proportions in any given series of pathological specimens. In Fig. 2 appropriate weighting factors have been introduced, which will take unique values for each individual pathologist in accordance with the varying nature of the particular selection chain along which his specimens have passed. The group of the population having neither ulcer nor cancer will be completely absent from such specimens and so will have weight zero. This is the only weight that the data of all pathologists will have in common.

It is now possible to restate symbolically the expected values for the *amalgamation ratio* and *amalgamation distribution ratio*, allowing for these weightings. It is important to realise that these new expressions are not valid for the population at large but only for a collection of pathological specimens, and this distinction will be emphasised by referring to them as observed ratios. On this new model, the *observed amalgamation ratio* will have an expected value, subject to sampling variations, of—

$$A_2 = \frac{w_7uc + w_8(1-u)\,(1-c)}{w_5u(1-c) + w_6c(1-u)}.$$

And the *observed amalgamation distribution ratio* will have an expected value, subject to sampling variations, of—

$$D_2 = \frac{w_7 uc}{w_8(1-u)\,(1-c)}.$$

The weighting complications are not yet ended, however. In the analysis so far, the expression " amalgamated lesions " has been used to imply no more than ulcer and cancer situated in the same region of the stomach, but all such combined lesions are not necessarily regarded by the pathologist as cases of " ulcer-cancer". Each will be judged on the personal criteria of the individual

		$w_0(1-\mathbf{U})\,(1-\mathbf{C})$
Ulcer		$w_1 u\mathbf{U}(1-\mathbf{C})$
	Ulcer	$w_2(1-u)\,\mathbf{U}(1-\mathbf{C})$
Cancer		$w_3 c(1-\mathbf{U})\,\mathbf{C}$
	Cancer	$w_4(1-c)\,(1-\mathbf{U})\,\mathbf{C}$
Ulcer	Cancer	$w_5 u(1-c)\,\mathbf{UC}$
Cancer	Ulcer	$w_6 c(1-u)\,\mathbf{UC}$
Ulcer and Cancer		$w_7 uc\mathbf{UC}$
	Ulcer and Cancer	$w_8(1-u)\,(1-c)\,\mathbf{UC}$

FIG. 2.

pathologist concerned over the period in question, and only a proportion of them will eventually be considered to fall into this histological group. Let us suppose that, in each individual series, k_1 represents this fraction of all pyloric amalgamated lesions thus designated " ulcer-cancer," and that k_2 represents the corresponding fraction along the lesser curve. These may be different, and must therefore be allowed for by appropriate separate weightings in the calculated ratios. Applying them to D_2 the final expression now becomes—

$$D_3 = \frac{k_1 w_7 uc}{k_2 w_8 (1-u)\,(1-c)}.$$

It will be seen that this expression represents the expected value on the null hypothesis of the " *ulcer-cancer* " *distribution ratio* as assessed by the pathologist, on the basis that " ulcer-cancer " is a component of the amalgamated lesions occurring at each site.

Similarly the ratio of pathologically diagnosed " ulcer-cancer " to all other associated cancer and ulcer lesions, which do not fall into this precise histological group, is now represented for each individual series by the expression—

$$A_3 = \frac{k_1 w_7 uc + k_2 w_8 (1-u)\,(1-c)}{w_5 u(1-c) + w_6 c(1-u) + (1-k_1)\,w_7 uc + (1-k_2)\,w_8 (1-u)\,(1-c)}.$$

It will be observed that this ratio includes for the first time in its denominator all the amalgamated lesions which are not included in the pathologist's " ulcer-cancer " category, as well as associated lesions occurring in different regions of the stomach. For this reason it carries a different pathological significance to that expressed by the previously defined *amalgamation ratio*. It now represents the true " *ulcer-cancer* " *ratio* on the null hypothesis—this term being used on the understanding that the existence of " ulcer-cancer " has yet to be proved. Its entity must be demonstrated by showing that the expected values we have quoted for the critical ratios are significantly different from the observed values. It will be seen that these two ratios, which were originally adopted because they had the desirable property of independence of the imponderables **U** and **C**, are nevertheless very obscure collections of weights resulting from the reference chain.

Practical application of the analysis.

The application of these weightings to the ratios commonly quoted in the relevant literature shows that, instead of being relatively simple, these are in fact extremely obscure. The first of them, the fraction of ulcers observed to show malignancy, denoted by the symbols $\frac{\mathbf{UC}}{\mathbf{U}}$ in our present system of classification and usually taken to estimate that ratio, is actually an estimate of the much more complex quantity,

$$\frac{k_1 w_7 uc\mathbf{UC} + k_2 w_8 (1-u)\,(1-c)\,\mathbf{UC}}{w_1 u\mathbf{U}(1-\mathbf{C}) + w_2(1-u)\,\mathbf{U}(1-\mathbf{C}) + w_5 u(1-c)\;\mathbf{UC} + w_6 c(1-u)\,\mathbf{UC} + w_7 uc\mathbf{UC} + w_8(1-u)\,(1-c)\,\mathbf{UC},}$$

whilst the second commonly quoted ratio, the fraction of all cancers observed to originate in ulcer, which is generally supposed to be represented by the ratio $\frac{\mathbf{UC}}{\mathbf{C}}$, is equally complex and is in fact—

$$\frac{k_1w_7uc\mathbf{UC} + k_2w_8(1-u)\,(1-c)\,\mathbf{UC}}{w_3c(1-\mathbf{U})\,\mathbf{C} + w_4(1-c)\,(1-\mathbf{U})\,\mathbf{C} + w_5u(1-c)\,\mathbf{UC} + w_6c(1-u)\,\mathbf{UC} + w_7uc\mathbf{UC} + w_8(1-u)\,(1-c)\,\mathbf{UC}.}$$

These ratios are even more obscure than those giving the values for A_3 and D_3, since there is no way of eliminating from them the additional quantities **U** and **C**. Each of them contains no less than twelve unknown weights, so that it is obvious that little reliance can be placed on estimates of the incidence of " ulcer-cancer " obtained in this way. Even if approximations could be made in respect of the other unknowns, the basic imponderables **U** and **C** still present a problem difficult of solution. The value of **C** might reasonably be estimated approximately, as has already been pointed out, from the Registrar-General's figures and the computable expectation of life with cancer of the stomach ; but no reliable estimate of **U** is available. Doll and Avery Jones (1951) have essayed this problem, realising the importance of obtaining some reliable approximation of it, but their results are open to criticism (Editorial, *Brit. med. J.*, 1951).

In view of the intractability of this approach, let us consider the somewhat simpler ratios A_3 and D_3, both of which suggest alternative modes of attack on the problem. The " *Ulcer-Cancer* " *Ratio*, A_3, although independent of both **U** and **C**, still contains 8 unknowns. Moreover, 2 of them, w_5 and w_6, are the weightings for referability of associated ulcer and cancer lesions in separate compartments of the same stomach—the pathological data for the frequency of which is scanty and its clinical recognition difficult. The " *ulcer-cancer* " *distribution ratio* D_3 is, however, more promising in that it includes only 6 unknowns. Essentially, this expression—

$$D_3 = \frac{k_1w_7uc}{k_2w_8(1-u)\,(1-c)}$$

is seen to be

$$= \left[\frac{k_1}{k_2}\right] \times D_2,$$

and it should be capable of detecting any significant aetiological factor between ulcer and cancer, conducing to the preferential choice of zones for development.

Despite the microscopical difficulties sometimes experienced in determining accurately the precise starting-point of pyloric growths, which may occasionally influence the opinion whether the lesion is an " ulcer-cancer " or merely an amalgamated one without aetiological connotation, it is probably true that any individual pathologist's criteria will be independent of the site of origin. If so, then $k_1 = k_2$ and the null hypothesis expectation for D_3 will be identical with that for D_2. Either of these two expressions the " *ulcer-cancer* " *distribution ratio* or the *observed amalgamation distribution ratio*, may therefore be used for testing the truth of this hypothesis. In both instances the same 4 unknowns are involved—u, c, w_7 and w_8—though these can be reduced mathematically to 3, since only the ratio $\frac{w_7}{w_8}$ of the 2 weights is required and not their separate ex-

plicit values. If the individual pathologist could be given this ratio of the weights concerned, and the values for u and c, he could then calculate the expected values for D_2 and D_3, against which the observed values for these ratios in his own series could be tested.

The evaluation of u and c from existing figures is, however, rendered impossible by the effect of the reference chain and the different criteria adopted for the boundaries of the various stomach zones, nearly all the published work on stomach pathology conflicting in this respect. Minor differences on the latter score between different series may be partially taken up in the weights w_7 and w_8, since all comparisons will be internal ones within each set of individual data, but the question is of academic interest only so long as marked reference chain effects persist. Separate independent research, deliberately designed to eliminate the effect of reference chains, would be necessary for their estimation. Moreover, even if the individual pathologist was supplied with values for u and c, he would still be unable to determine whether his observed values for D_2 and D_3 differed from the null hypothesis expectation for these ratios. The ratio $\frac{w_7}{w_8}$ is inherently a property of his own series, representing the effect of the reference chain operating in his particular case. It can therefore only be determined by using his own observed frequencies for u and c and checking them against those established through such an independent survey. Manifestly, he cannot use his own data both to establish the expected value of the expressions D_2 and D_3, and then to see if his observed values differ from expectation. The position, from the point of view of the individual pathologist, is that significant departures in this respect from the null hypothesis predictions are inextricably confused with the effect of the reference chain ; and the same applies, even more, to the ratios A_2 and A_3, which are under the influence of the reference chain to a greater extent.

This is a point of paramount importance. Its implication is that no pathologist, however competent, and no consensus of opinion among pathologists, however widespread, can refute the null hypothesis on the basis of existing figures.

It follows inevitably that the solution to this problem of the incidence of "ulcer-cancer" must await a research project specifically designed to remove the insuperable obstacle facing the individual pathologist—the effect of the reference chain represented by the weights w in our analysis. The results of such an investigation could then be evaluated by means of the ratios A_1 and D_1, which only involve the site distribution factors u and c. It would be essential for this purpose to establish some sufficiently accurate convention defining the boundaries between the various stomach zones. Admittedly, these are not always easy to demarcate in practice, but the contrivance of some effective standard in this respect ought not to present insuperable difficulties.

The most hopeful way of establishing reliable values for u and c would appear to be by planned, clinical research at a number of large centres. The assiduous co-operation of all the local general practitioners surrounding these centres would be an essential, so that every case of a suspected stomach lesion reaches them at the earliest possible moment. By avoiding the symptomatic treatment of such patients for prolonged periods at home before reference for consultative opinion, accurate site diagnosis of the lesion would be rendered more certain. There would also be less chance of gastric ulcers healing undiagnosed ; and less

possibility of secondary carcinoma, which may have arisen at or near the site of a prior ulcer, developing sufficiently to obscure the primary pathology. Every patient reaching such a centre would have to be most carefully investigated and kept under constant review, radiological and gastroscopic checks being repeated at adequate intervals. All cases of proven gastric ulcer would have to be followed up in the out-patient department until healing was complete, and any failing to respond to medical treatment, together with every case of suspected neoplasm, pursued vigilantly to either theatre or post-mortem room. Similarly, all patients admitted to such centres as emergencies, with complications such as haematemesis, perforation or acute exacerbation of an ulcer, would have to be transferred to the out-patient department for further review after their discharge.

Projected clinical research of this type would, of course, besides entailing the wholehearted assistance of the neighbouring general practitioners, require a great volume of routine follow-up work and the effective co-operation of the patients concerned. But, only by such measures, can the diagnostic net be thrown wide enough to be able to detect the presence of a secondary neoplasm adjacent to the gastric lesion at the critical moment for accurate diagnosis. The research would have to be based on several centres, whose respective findings could finally be checked for statistical homogeneity, and these centres ought to be carefully selected. They should be situated at large-population centres which are growing, to minimise loss of patients during prolonged observation from drift to other areas ; and in provincial towns rather than in very large cities, where it might be difficult to trace their admission to, or treatment at, a large number of alternative hospitals for a gastric emergency or some intercurrent disease.

If the establishment of such centres were practicable, it might be possible to eliminate the reference chain weights w for all practical purposes, and thus arrive at reliable values for the site distribution factors u and c. There are, of course, dangers of leakage in the procedure outlined. In addition to the inevitable loss of some patients during the prolonged observation necessary, there is the certainty that some cases would not reach autopsy. Moreover, there will be a fraction of the population who will not seek any medical advice until the disease has progressed too far for any statistical value to be elicited regarding the site diagnosis. This is not the place to discuss the details of such an investigation, but the essential problem regarding such losses is not so much their magnitude as whether they are likely to introduce bias in the determination of u and c.

If unbiased estimates of u and c could be obtained in this way, the data collected would suffice to test the null hypothesis expectations. Cases with single lesions could be used to provide values for these quantities, and thus for the expected values of A_1 and D_1, of a high degree of accuracy deriving from the large number of observations on which they would be based. Cases with double lesions would then give an adequate check on such results, using the incidence figures of ulcer and cancer in different stomach zones for this purpose in addition to those of amalgamated lesions in the same zone. As such a challenge to the null hypothesis involves absolutely no assumption of the pathological criteria for " ulcer-cancer ", the establishment of this entity by so direct a statistical investigation would be highly significant and the direction of departure from the null hypothesis expectations by both amalgamated and separate lesions would be most instructive. It would, in any case, still be open to the pathologists at the research centres, providing that they can agree the criteria for the establish-

ment of this entity, to continue to make diagnoses of " ulcer-cancer " and, on the assumption that $k_1 = k_2$, to dispute the null hypothesis in terms of the " *ulcer-cancer* " *distribution ratio*, this expression—

$$D_3 = \frac{uc}{(1-u)(1-c)},$$

now having the same expected value on the null hypothesis as D_1, once the effect of the weights has been removed, though its actual significance is different.

CONCLUSIONS.

By investigating this problem along the lines suggested, it ought to be possible eventually to accumulate sufficient evidence to confirm or refute the null hypothesis that " ulcer-cancer " is not a true entity. Moreover, if it should be shown that this lesion is indeed a pathological actuality, it should also be possible to determine a reasonable approximation of its incidence in a statistically sound manner—an impossible task on the existing evidence. Clinicians would then no longer be condemned to wander in a maze of conflicting pathological evidence regarding the ultimate prognosis of chronic gastric ulcer, but would be given an answer which would assist them materially in their treatment of this disease. Until that time comes, however, it seems that there is no more cogent basis for the assumption that malignant degeneration is a common event in gastric ulcer than argument by analogy from carcinomatous changes consequent on chronic inflammation elsewhere in the body.

SUMMARY.

1. An investigation has been made into the existing evidence for secondary neoplastic change in chronic gastric ulcer.

2. Insufficient purely clinical data exists to justify the frequent diagnosis of " ulcer-cancer " in its accepted connotation, and until pathologists in general can agree their criteria for the microscopical establishment of this entity, the histological evidence for such malignant change cannot be accepted at face value.

3. Furthermore, it is suggested that this concept of " ulcer-cancer " as a not uncommon sequel to longstanding gastric ulceration is equivocal, probably having arisen initially by analogy with similar transformation in chronic inflammatory tissue elsewhere in the body.

4. Since a dialectic approach of this kind may lead to most fallacious conclusions, this investigation has accordingly been based on the postulate of a null hypothesis, to the effect that " ulcer-cancer " does not exist as a true entity in the aetiological sense.

5. The construction of a statistical model of the problem on these lines shows, however, that the figures commonly quoted in the literature for the incidence of " ulcer-cancer " are invalid and illusory. So many imponderables are concerned that attempts to calculate such ratios on the basis of existing data must be foredoomed to failure.

6. A new approach to the problem is therefore suggested, designed to remove the effects of these unknowns. It entails the early reference to special research

centres of all patients with symptoms suggestive of any gastric pathology for careful investigation and subsequent observation over an adequate period of time, and should be capable of yielding figures of definite statistical value.

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CUTANEOUS ANGIOSARCOMA WITH METASTASES.

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Received for publication June 7, 1952.

SCEPTICISM is a healthy response to diagnosis of any tumour as angiosarcoma. To dispel all doubts, full necropsy and thorough histological examination of all lesions with indisputable evidence of the vasoformative nature of the tumour and proof of metastasis are essential. The case to be described fulfils these requirements.

History.

Mrs. E. H—, aged 69, was admitted to the Royal Cancer Hospital on July 31, 1950, complaining of an ulcer on the left temple. One year previously she had been bitten about the face, forehead and scalp by gnats. One of these, on the left temple, had blistered and discharged pus and blood and, in spite of administration of penicillin and one treatment by high voltage radiation, an indolent ulcer formed. In June, 1950, swelling and bluish discoloration had appeared overnight in her right cheek.

Local examination.—There was a superficial ulcer (1·5 cm. diameter) with hard raised margins, resembling rodent ulcer, on the left temple. The right cheek was oedematous and showed diffuse induration and blue discoloration, which was fading.

The ulcer was excised and a biopsy was taken from the right cheek. During her short stay in hospital the incision at the temple healed and the discoloration and induration of the right cheek were receding. She was discharged on August 14, 1950.

Histology (Professor R. A. Willis).—(1) Excised ulcer left temple: "The skin is infiltrated by mingled chronic inflammatory cells and actively proliferating fibroblasts. In spite of the cellular pleomorphism and many mitoses, the lesion is almost certainly only inflammatory." (2) Skin from right cheek: "Fragment of skin shows inflammatory changes and compact masses of large cells of uncertain nature just beneath the epithelium."

A note was added suggesting a gnat-bite granuloma might account for the ulcer on the temple, and that the biopsy from the right cheek was too small for diagnosis.

In November, 1950, the left temple had healed. The right cheek had again become swollen from an extravasation of blood producing a haematoma ($3\frac{1}{2}$ in. long $\times$ 2 in. wide) with its medial border along the naso-labial fold. There was no ulceration, but three small blebs were present along the upper margin. At this stage full haematological investigations were carried out to eliminate any

blood disorder. They were negative. By March, 1951, her condition had become more serious. The right cheek was firmer, more swollen, and reddish-blue discoloration had spread over a much wider area, including the orbital tissues (Fig. 1). Cords of induration could be felt in the skin beyond the margins. A course of high-voltage radiation was given with excellent results. The swelling and induration decreased and the edges receded. A further biopsy was taken at this stage.

Histology (Dr. J. W. Whittick).—" Infiltration of the dermis by cellular malignant growth consisting of large cells of fairly uniform type which appear to be vaso-formative. The appearances are very suggestive of angiosarcoma."

From this time, despite palliation with high and low voltage therapy and radio-active phosphorus (P 32), her general condition progressively deteriorated and the local condition was a succession of minor remissions and severe exacerbations. Eventually the lesions spread over the right side of the face, the anterior half of the scalp and down the left side of the face (Fig. 2). Enlarged tender lymph nodes appeared in both supraclavicular fossae and posterior triangles of the neck. She died on September 30, 1951.

Necropsy.

(Thirty-four hours after death): Adequately nourished, but not obese, elderly female. Extending over the right side of the face, from the lower border of the mandible, across the forehead and anterior half of the scalp and down the left side of the face to the upper margin of the mandible, were numerous irregular, finely nodular, red elevations of various sizes (largest, 1·5 cm. main diameter). They were set closely together, leaving little normal skin, and had become confluent over the scalp, where the epidermis was ulcerated and covered by yellowish crusts. At the margins the red areas were smaller and flatter. Many lymph nodes in the cervical regions, supraclavicular fossae and posterior triangles were enlarged (up to 2·0 cm. main diameter), soft, dark red and cystic. On cutting into them fluid blood escaped, leaving soft, reddish-brown tissue. On the inferior surface of the tongue was a small, flat, dark red, circular area (0·4 cm. diameter). The brain, meninges, middle ears, pharynx, larynx, trachea, oesophagus, thyroid and breasts were normal. No fluid was present in either pleural cavity, but there were adhesions on the right side both between lobes and between lung and chest wall. The bronchi were normal. On the surface and in the substance of both lungs were numerous discrete, firm, red, spherical nodules (up to 3·0 cm. diameter) whose cut surfaces resembled blood clot. Other parts of the lungs were oedematous. The paratracheal and mediastinal lymph nodes were greyish-black and slightly enlarged. The thoracic duct was normal. The heart was enlarged from dilatation and hypertrophy of the left ventricle. No fluid was present in the peritoneal cavity. On the surface of the right lobe of the congested liver was a small, flat, dark red area (0·2 cm. diameter). The common bile-duct was dilated and contained several small pigment calculi. The spleen was small, and on its surface was a cyst (1·2 cm. diameter) containing clear fluid. The stomach, intestines, pancreas, adrenals, kidneys, ureters, bladder, uterus and appendages were normal. The skull was partially infiltrated, but nowhere penetrated, by soft red tumour tissue from the overlying affected scalp. The ribs, sternum and thoraco-lumbar vertebral bodies were normal.

Histology.

The histology of sections from the face, scalp, cervical lymphatic glands and lungs, all of which contain angiosarcomatous tissue, will be described. The reddish areas in the tongue and liver are confluent petechial haemorrhages. The lesion in the spleen is a simple intra-capsular cyst with fibrous walls; no angiomatous tissue is present. Significant abnormalities are not present in other organs.

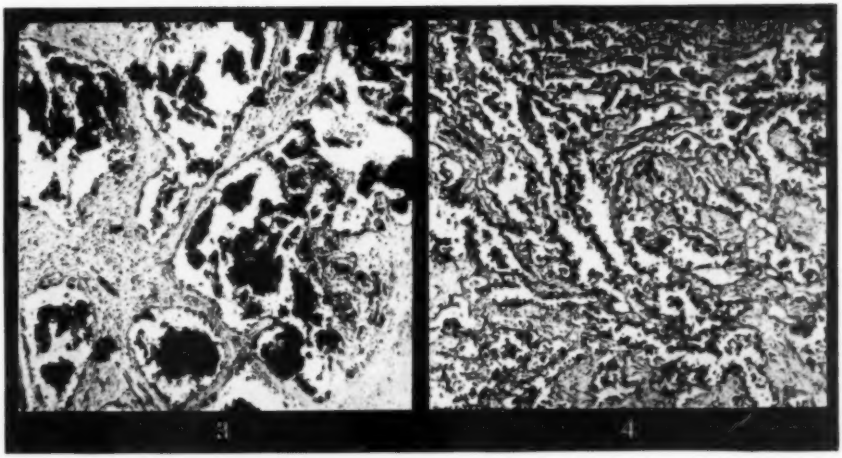
Face and scalp (Fig. 3, 4).—A few hair follicles, sweat glands and arrector pili muscles escape the extensive replacement of the dermis and subcutaneous fat by well differentiated angiomatous tissue. Communicating cavernous vascular spaces, filled with blood and lined by a single layer of endothelium, are separated by varying thicknesses and densities of collagenous tissue. In other parts, particularly where fat is infiltrated, capillary vessels are set in looser fibrous tissue. In places tumour blood-vessels lie against the basal layer of the epidermis, which elsewhere is ulcerated, or else thickened with papillae projecting down into the perivascular fibrous tissue. Histiocytes containing haemosiderin are ubiquitous and numerous. Irregularly and infrequently dispersed through the tumour blood-vessels are compact balls of swollen endothelial cells whose cytoplasm is faintly eosinophilic and sometimes indefinitely outlined. The nuclei are pale-staining, vesicular and variable in size: in shape spherical, ovoid or indented. Mitotic figures are few, but within such aggregations capillary channels are forming by separation of the cells. At one point in the scalp perineural lymphatics are occupied by capillary-forming endothelial cells (Fig. 5).

Cervical lymph glands.—Large, communicating, thin-walled vascular channels, filled with blood, replace much of the lymphoid tissue. Aggregations of incompletely differentiated endothelial cells, identical with those in the tumour areas of face and scalp, though more numerous, are present within these spaces, particularly at the sites of the peripheral sinuses (Fig. 6). In addition, small fragments of tumour are present within several of the afferent lymphatics to the nodes (Fig. 7).

Lungs.—The nodules in the lungs are composed of vascular spaces lined by a single layer of endothelium and filled with erythrocytes and blood clot. In parts the spaces roughly conform to the shape of the alveoli, by whose walls they are supported. Where the alveolar walls are intact, at the periphery of the nodules, the margin is clearly demarcated from normal lung tissue (Fig. 8). In other places, both alveolar walls and the walls of the vascular spaces have broken down

EXPLANATION OF PLATES.

FIG. 1.—Angiosarcoma: the primary lesion before irradiation.
FIG. 2.—Angiosarcoma: the lesion one month before death.
FIG. 3.—Tumour of face: large vascular spaces lined by a single layer of endothelium. H. & E. × 65.
FIG. 4.—Tumour of face: smaller vascular spaces in fibrous stroma. Gordon and Sweet. × 160.
FIG. 5.—Tumour of scalp: angiosarcomatous tissue within a perineural lymphatic. H. & E. × 190.
FIG. 6.—Cervical lymph node: tumour tissue within peripheral sinus. H. & E. × 120.
FIG. 7.—Cervical lymph node: tumour cell group within blood-containing afferent lymphatic. H. & E. × 180.
FIG. 8.—Lung metastasis: cavernous tumour vessels at periphery of deposit. H. & E. × 120.
FIG. 9.—Lung: tumour tissue within a pulmonary artery. H. & E. × 90.
FIG. 10.—Lung: tumour tissue within a smaller pulmonary blood-vessel. H. & E. × 190.



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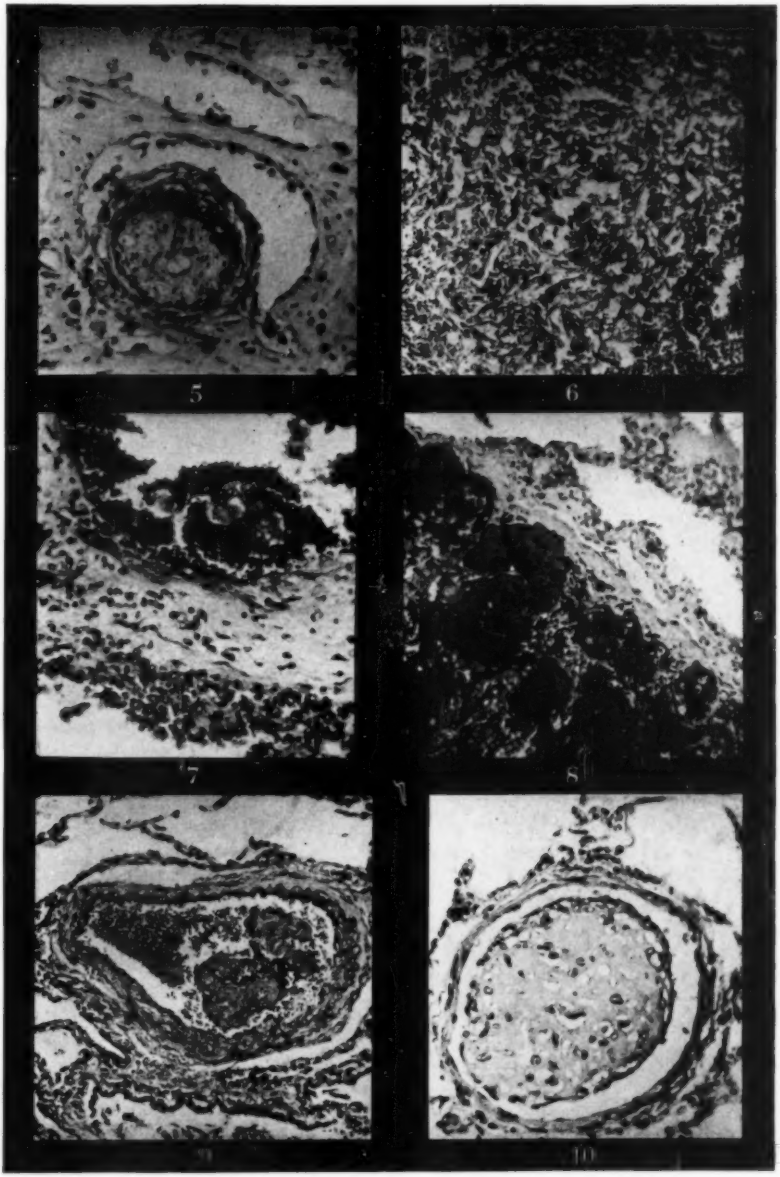

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so that there is variation in size and shape of the spaces and, at the periphery, the edge is indefinite and blood seeps into adjacent alveoli. It appears that infiltration is achieved by malignant endothelium extending into and lining alveoli, which become distended with blood, rupture, and so provide communication with fresh alveolar spaces. In this way lung tissue is gradually infiltrated, leaving large lakes of blood in which lie swollen endothelial cells, singly or in groups. Irregularly dispersed throughout the lesions, but not numerous, are compact aggregations of swollen endothelial cells similar to those described in the face and lymph nodes. Fragments of tumour tissue are also present within the lumen of small pulmonary arteries and veins (Fig. 9, 10).

Conclusion.

Angiosarcoma of the right cheek spreading by contiguity over the face and scalp with embolic metastases in the cervical lymph glands and the lungs.

DISCUSSION.

Willis (1934), in a critical review of the subject of metastases from angioblastic tumours, besides casting doubt on the accuracy of the diagnosis in many reported cases, makes these points : (1) Multi-focal benign angiomatoid lesions occur both in children and adults. (2) Some reported cases have a " system " distribution and involve combinations of spleen, liver, bone marrow and lymph nodes. These may be multi-focal lesions, not necessarily developmental, rather than metastases from a primary tumour. (3) Cases have been recorded where angiomatoid formations appear to have genuine neoplastic qualities and intravascular extensions—observations which " indicate at least the setting of the stage for the occurrence of haemic embolic metastases."

In the present case full necropsy with multiple blocks from all lesions revealed no other tumour, and the histological appearances are not suggestive of either vascular sarcoma of non-angiomatous type or haemorrhagic adenocarcinoma. The lesions consist of vasoformative tissue characterised by aggregations of imperfectly differentiated, but clearly recognizable, endothelial cells with transitions to well-formed vascular spaces of various sizes and shapes invading the tissues locally. The sites of the lesions (face and scalp, cervical lymph nodes and lungs) and the sequence of events suggest a metastasizing neoplasm, and as, in addition, tumour tissue is demonstrable within lymphatics and blood-vessels in the lines of communication, this becomes a certainty.

The histological structure of the lesions is deceptive. All are predominantly benign in appearance, and might be regarded as " simple angioma " but for occasional aggregations of swollen endothelial cells which show variation in size and shape of their nuclei. Robinson and Castleman (1936) reported a case in which a tumour in the breast retained its histologically benign angiomatous appearance after recurrence, but metastases in the other breast showed areas suggestive of malignancy. Shennan (1914) recorded a case in which a tumour of the superior mediastinum was associated with multiple tumours in many tissues. Histologically all were benign, but he illustrated apparently simple angiomatous tissue penetrating the pulmonary vein. Borrmann (1907) described a case in which an angioma in the skin of the breast, benign in histological structure in all specimens, recurred four times after surgical excision. The patient died, and at necropsy

there were multiple metastases in the lungs and one in the gluteal region. Histologically all were as benign in structure as the original lesion. He interpreted his case as a benign angioma producing metastases, and separated it sharply from a case described by Theile (1904) in which all the lesions showed sarcomatous areas. His interpretation is not acceptable in pathology, and the histology of the lesions in Shennan's (1914) case was no less benign, yet penetration of a pulmonary vein was found. Shennan suggested that our criteria of malignancy were at fault in regarding such lesions as histologically benign. Livingstone and Klemperer (1926) concluded that the lesions in these cases were not as benign histologically as the authors believed, and they thought that all showed atypical areas suggestive of malignancy. Stout (1943) confirmed this, and considered the same applied to the original lesion in Robinson and Castleman's (1936) case. However, in this connection it is well known that other malignant tumours and their metastases sometimes show deceptively benign histological appearances (e.g., chondrosarcoma and carcinoma of prostate, thyroid or kidney).

The present case seems to be of the same nature as these and, in addition, has features similar to some others in which the histological structure was more malignant. Kettle (1918) described an angioblastic tumour of the leg with metastases in the inguinal lymph nodes, Ulrich (1921) a similar tumour of the lumbar muscles with metastases in the lungs, and Downing and Mallory (1930) multiple angiomatoid lesions appearing in successive crops over an area which had been burnt some months previously. In the last two cases intravascular extensions were present, which strongly suggests that the lesions other than the primary were blood-borne metastases. Mallory (1914), however, states that some cavernous haemangiomas not only arise in veins but permeate their walls and, at various places, rupture them, infiltrate the tissues and produce lesions which, while simulating metastases, are, in reality, in contiguity. This must be unusual, and would clearly be an incorrect interpretation of some of the cases cited where the lesions are far apart and particularly where peripheral lesions are associated with pulmonary ones. On the other hand, if benign angiomatous lesions extend into the lumina of veins, it cannot be concluded from observing intravascular extension alone that multiple lesions are metastatic rather than multifocal.

In the present case, however, tumour tissue, not in contiguity with large masses, is demonstrable within pulmonary vessels and, of special interest, within perivascular lymphatics and afferents to cervical lymph nodes. The only acceptable explanation of such findings is that the lesions in the cervical lymph nodes and lungs are embolic metastases.

This case does not appear to shed any new light on those angiomatous conditions having a "system" distribution described by Theile (1904), Jores (1908), Wright (1928) and De Navasquez (1936). In all these the authors described primary angioblastic tumours of the spleen with metastases in the liver. In Theile's case there were additional lesions in the lungs and stomach. Willis (1948), while conceding these authors' interpretations may have been correct, points out the possibility of the lesions in their cases being multifocal in origin. Evidence suggestive of such cases being malignant metastasizing neoplasms (local infiltration, poor differentiation, mitoses and intravascular extensions) is not sufficient to classify them, with certainty, as such. The case described by Wright, however, possessed all these features and, in addition, the hepatic lesions were not only very numerous but varied considerably in size, suggesting they had not all been present for the

same length of time. It is considered that Wright's interpretation of his case is fully substantiated.

Stout (1943) mentions that local infiltration of the tissues in angiosarcomatous lesions is achieved by sprouting of capillaries in much the same way as occurs in granulation tissue. This is confirmed in this case. The budding capillaries are supported in a loose fibrous stroma except in some of the pulmonary lesions where alveolar walls afford the support. It seems, however, that unless a fibrous stroma is acquired, the thin endothelial walls between the spaces rupture and lakes of blood are left. This has occurred in most of the pulmonary lesions.

Kettle (1918) described the formation of lumina in cords of sarcomatous endothelium by the coalescence of vacuoles which appear in the cytoplasm of the central cells. He considered this a distinguishing feature of vasoformative tissue. In the present case, however, vacuolated cells were irregularly dispersed, and any relationship between vacuolation and lumen formation was not apparent. Vascular spaces seemed to form by cleavage of endothelial cells when in compact groups, in the same way as alveolar spaces would appear on inflating a collapsed lung.

SUMMARY.

A case is described of primary cutaneous angiosarcoma of the face with metastases in cervical lymph nodes and lungs in a woman aged 69. Tumour tissue was found within perineural lymphatics of the scalp, in the afferent lymphatics of cervical lymph nodes and within pulmonary blood-vessels.

I am indebted to Dr. J. W. Whittick for his help and interest in the preparation of this paper, to Mr. A. H. Hunt and Professor D. W. Smithers for access to the clinical notes and permission to publish the case, to Mr. G. C. Chadwin for the histological preparations and to Mr. L. A. Cowles for the photomicrographs.

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THE FREQUENCY OF MITOSIS IN THE EPIDERMIS OF NORMAL AND TUMOUR-BEARING MICE.

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Received for publication August 8, 1952.

ALTHOUGH wasting and debility are common clinical findings in patients suffering from cancer, the nature of the relationship is still very obscure. Some authors (Willis, 1948) believe that the association is mainly indirect, and that the nutritional features result chiefly from anorexia, ulceration and infection, to which, with cancers of the alimentary tract especially, are added disordered digestion and absorption of food. Others consider that the connection may be more direct, perhaps through metabolic competition for circulating nutrients, and in recent years animal experiments have lent some support for this hypothesis. A reduction in plasma albumin concentration has been noted both in cancer patients and in tumour-bearing animals (Abels, Rekers, Binkley, Pack and Rhoads, 1942; Siebert, Siebert, Atno and Campbell, 1947; Huggins, 1949). An early fall in the blood haemoglobin level (Greenstein, Andervont and Thompson, 1942; Taylor and Pollack, 1942; El Mahairy, 1950) and marked reductions in tissue enzyme activity, e.g., in liver catalase, in organs far distant from the growth have been similarly recorded (Greenstein, 1947).

While there is no doubt that a growing cancer often exerts widespread effects on general metabolism, no study has yet been made of any process common to both normal and tumour cells in which any such metabolic competition might be expected to disclose itself. With this in mind, therefore, it seemed profitable to make a comparative study in normal and tumour-bearing animals of the occurrence of mitosis—a phase of the growth process which to the best of our present knowledge presents similar features in both types of cell. Accordingly, experiments have been undertaken to determine whether the presence of a growing transplanted tumour in a mouse is associated with any alteration in the frequency of mitotic cell division in normal host cells, as this is exemplified in the physiological regeneration of the epidermis.

MATERIALS AND METHODS.

Owing to the known liability of the mitotic activity of epidermal cells to be affected by such factors as sex (Bullough, 1943), age (Bullough, 1949*a*), nutrition (Bullough and Eisa, 1950), time of day (Carleton, 1934; Cooper and Franklin, 1940; Blumenfeld, 1943; Bullough, 1948), as well as by such environmental conditions as temperature and humidity (Bullough, 1949*b*), precautions have been taken to minimize these recognized sources of variation.

Animals.

To eliminate variations that might arise from the use of different strains, a sub-strain C of the Bagg albino mouse, maintained by brother-sister mating, has

alone been used. Because of the wide range in mitotic activity in the epidermis of female mice at different phases of the oestrus cycle, only males were employed. At the beginning of each experiment they were sexually mature animals, 3 to 4 months old.

Diet.—Throughout the experiments the mice were given the semi-synthetic diet described by Folley, Henry and Kon (1942), with appropriate vitamin supplements. In some experiments the protein content was doubled at the expense of the carbohydrate ; this " high-protein " diet contained 40 per cent of casein. With the aid of a little water all the ingredients were mixed into a stiff paste which the mice ate readily. Food and water were always available.

Maintenance.—When the experiments were begun, it was thought that with so thin a structure as the pinna, the ambient temperature might modify importantly the rapidity of epidermal mitotic activity. The mice were accordingly maintained during the experiments in an incubator thermostated at 25° C. In a paper published during the course of the present work, Bullough (1949*b*) stated that changes in temperature within the range normally found indoors in this country had no noticeable effect on mitotic activity in the skin of the ear. The use of the thermostat was retained, however, all the animals being kept in divided cages with a normal control and a tumour-bearing mouse sharing a compartment.

Tumour.

In all the experiments the Imperial Cancer Research Fund Sarcoma S 37 was used. This anaplastic tumour grows readily in the strain of mouse used ; it has a high percentage of " takes," and shows very little tendency to regress when once established. It was maintained by successive transplants at 3-weekly intervals. Inoculations of both maintenance and experimental mice were made into the subcutaneous tissues of the flank—small pieces of tumour were inserted through a wide-bore needle.

Preparation of epidermal specimens.

Samples of epithelium were obtained from the pinna of all mice, control and tumour-inoculated, at the beginning of each experiment. The second specimen was taken from both series of animals at the time that the tumour was judged to have grown to about 10 per cent of the total weight of the mouse.

All specimens were taken at 10 a.m. to avoid variations due to the diurnal cycle (Bullough, 1948). Five minutes beforehand the pinna was epilated with barium sulphide paste. Immediately after its removal the piece was trimmed all round in order to expose a raw edge which facilitated the penetration of the cold 1 per cent acetic acid solution in which it was at once immersed. After 1½ to 2 hours in this solution (the time depending on the size of the piece), it was possible to slide the epithelium from both sides of the underlying connective tissues. The epithelial sheets so obtained were flattened on the surface of water and taken up on small pieces of filter-paper. After it had been covered with a similar piece of filter-paper the whole was moistened with fixative, compressed lightly between two coverslips held together with an elastic band, and immersed in fixative for at least an hour. The fixative used was a mixture of one part of glacial acetic acid with two parts of absolute alcohol ; exposure to this solution for several days did not greatly harm the cells, but a period of not more than 24 hours gave optimal results. Staining was carried out by Feulgen's method. To protect the delicate sheets of epithelium

during the stage of hydrolysis, it was necessary to retain them between the filter-papers. The "sandwich" was therefore removed intact from the coverslips, washed free from fixative in 33 per cent alcohol followed by water, and then soaked for a short time in N/1 HCl before it was replaced between the coverslips and their rubber band. The "sandwich" was then placed for 4 minutes in a pot of N/1 HCl kept at 60° C.

The epithelium could now be separated from its protective papers and placed directly in the staining solution, which was prepared by reducing basic fuchsin with bisulphite according to the method of de Tomasi (Darlington and La Cour, 1942). After about 1 hour the maximum degree of staining was reached, and a subsequent washing for a similar period in SO_2 water was needed to remove unchanged stain which would otherwise have become re-oxidized in the later treatment. The stained sheets were now dehydrated in alcohol, cleared with xylol or cedar wood oil and mounted in either canada balsam or thick cedar wood oil. In a satisfactory specimen only the nuclei were stained.

Estimation of Mitotic Activity.

The mitotic activity of the epithelium was estimated by counting the number of mitoses seen in 100 arbitrarily-chosen microscopic fields viewed at a known magnification. The field studied was delimited by means of a disc with a small central square hole inserted into the eyepiece of the microscope A $\frac{1}{12}$-inch oil immersion objective gave a magnification of 540 with adequate definition and good depth of focus. On average, about 200 nuclei were visible in each field; control of the preparation with a mechanical stage enabled fields to be picked at random without duplication. For each specimen, fields were taken from all portions in order to exclude variations in mitotic activity in different areas of the ear surface. Duplicate counts on specimens showed that consistent results were obtained by viewing 100 such fields. Any improvement from counting 200, as Knowlton and Hemplemann (1949) did, was too slight to be significant and, as Knowlton and Widner (1950) found later, was incommensurate with the extra time required.

RESULTS.

1. *Changes in the mitotic activity of ear epithelium in tumour-bearing mice.*

The results of three similar experiments which show the general effect of the growth of the tumour on the mitotic activity of the epidermal cells of the pinna are given in Table I. In Experiment 1 the mice were maintained on the basic semi-synthetic diet which contained 20 per cent of casein; in Experiments 2 and 3 the protein content of this diet was raised to 40 per cent, the carbohydrate content being reduced proportionately.

These experiments show that the growth of an implanted tumour to about 10 per cent of the mouse's weight is accompanied by a fall in the frequency of the mitoses in the epidermal cells of the pinna, and that this decline takes place on both the ordinary semi-synthetic and the "high-protein" diets. With the control mice, maintained under identical conditions, no such significant change in the mitosis rate took place; although the mean frequency was rather lower at the end than at the start, the difference was small in comparison with its standard error. The change observed in the tumour-bearing animals thus seems to be attributable to changes in metabolism induced by the enlarging neoplasm.

TABLE I.—*Mean Numbers of Mitoses, per 100 Microscope Fields, in the Epidermis of Control and Tumour-Bearing Mice at the Time of Implantation (Start) and at the Time that the Tumours had reached about 10 per cent of the Mouse's Weight (End).*

Experiment number.	Controls.		Tumour-bearing.	
	Start.	End.	Start.	End.
1*	29·0 (3)	18·0 (4)	17·6 (8)	5·5 (6)
2†	18·3 (3)	22·6 (5)	20·8 (5)	9·6 (5)
3†	16·7 (7)	14·8 (5)	18·6 (5)	7·2 (5)
Means of totals	20·0±1·7 (13)	18·5±1·9 (14)	18·8±1·2 (18)	7·3±1·7 (16)
Differences	1·5±2·5		11·5±2·1	

* Mice on semi-synthetic diet.
† Mice on "high-protein" semi-synthetic diet.
Figures in parentheses show the number of mice used.

2. *The relation of the reduction in the mitotic activity of epidermal cells in tumour-bearing mice to changes in their weight and food intake.*

Since it is possible, as some authors have suggested, that systemic effects in persons or animals bearing tumours may be due more to depression of appetite and lowered food intake than to the direct competitive effect of the tumour itself, some experiments were performed to determine how far such indirect factors may be the cause of the reduction in mitotic activity. In the first of these, the results of which are shown in Table II, the mice were weighed at the time of implantation and again when they were sacrificed at the end of the experiment in order to determine whether changes in mitosis rates were associated with significant losses

TABLE II.—(a) *Weights of Tumour-Bearing Mice at the Times of Implantation and Death;* (b) *Tumour Weight as Percentage of Body Weight at Death;* and (c) *Mean Numbers of Mitoses in Epidermis per 100 Fields at Times of Implantation and Death.*

Type of diet.	Weight of mouse (g.).		Weight of tumour (percentage of body weight).	Number of mitoses (per 100 fields).	
	Implantation.	Death.*		Implantation.	Death.
Semi-synthetic	28·9	29·1	14	12	4
	25·4	25·0	14	16	4
"High-protein"	27·0	26·3	13	22	6
	25·4	25·8	14	24	13
	23·7	24·8	10	26	3
	24·5	26·0	11·5	14	3
	27·0	26·2	8	22	26
	24·5	26·2	7·5	18	8
	24·0	26·0	11	24	8
	33·4	38·6	13	20	2
	24·2	24·0	12	18	2
	27·9	27·6	9·5	24	18
	28·0	27·8	11	16	6
	27·2	26·2	12	15	8
Means and S.E.	26·5±0·7	27·1±0·9	11·5	19·4±1·2	7·9±1·9
Difference and S.E.				11·5±2·2	

* Weights of carcasses after removal of tumour.

in weight. Since the tumours had reached about 10 per cent of the animal's body weight at the time of death, the neoplastic tissue was resected before the final carcass weight was determined.

From Table II it can be seen that even when the tumours had reached this large proportion of the body weight, the average carcass weight had not fallen from the time of implantation. Although there was no sign of wasting in these tumour-bearing mice, and their behaviour continued to be typically active, the frequency of mitosis in the epidermal cells had fallen significantly.

A further test of the inanition theory of reduction of mitosis lay in determining whether there was any correlation between the change in the weight of the mouse in the final 24 hours and the frequency of mitosis. Since the mouse has a very high food intake—15 to 20 per cent of its body weight daily—any abstinence from eating is quickly revealed by a loss of weight. In Table III the control and tumour-bearing mice have been subdivided into two groups on the basis of change of weight in the preceding 24 hours, and the mean number of mitoses per 100 fields of the ear epidermis recorded. From these figures it can be seen that small gains or losses in weight during the previous day have no significant influence on the frequency of mitosis—indeed, in both control and tumour-bearing groups, the mice that had a small loss of weight tended to have slightly higher mitosis counts.

TABLE III.—*Change of Weight of Mice in the 24-Hour Period immediately preceding Ear Sampling and the Number of Mitoses per 100 Fields in the Epidermis of Control and Tumour-Bearing Mice.*

	Control.			Tumour-bearing.	
	Gain.	Loss.		Gain.	Loss.
Number of mice in group	20	14		7	9
Mean gain or loss (g.)	0·7	0·5		0·8	0·4
Mean and S.E. of number of mitoses per 100 fields	16·8±1·8	20·9±0·7		6·7±3·3	7·9±1·7
Differences and S.E.	4·1±1·9			1·2±3·7	

DISCUSSION.

From the experiments described above it seems that the growth of a transplanted tumour in a mouse results in some metabolic disturbance which affects adversely the ordinary physiological regeneration of the host's epidermal cells. The fall in the mitosis rate in this epithelium appears to take place in spite of the fact that the animals show no sign of deterioration in their general condition. Even when the tumours attain a weight of about 10 per cent of that of the host, the mice seemed normally active and consumed as much food daily as their controls. Moreover, their body weight at death, after exclusion of the tumour, had altered little from that at the time of implantation. These general findings are in conformity with those of Sherman, Morton and Mider (1950), who observed that in tumour-bearing rats the nitrogen required for the nutrition of the tumour was derived from the diet until the growth reached about 10 per cent of the host's body weight, and that it was only after this proportion had been exceeded that this requirement was obtained at the expense of wasting of the body tissues. As long as this ratio of tumour to body weight is not exceeded, therefore, it seems that the reduction in the frequency of mitosis in the epidermis takes place independently of any metabolic disturbance that interferes overtly with the usual physiological

activities of the host animal. It is difficult, consequently, to attribute the decreased rate of multiplication of the epidermal cells in our mice to any change comparable with the intoxication and cachexia often present in the later stages of neoplastic disease in man.

An alternative explanation for our observations is that during the growth of such implanted tumours nutrient substances essential for cell division may be diverted from normal to neoplastic cells to the detriment of the former. During periods of nutritional stress, such as severe malnutrition or pregnancy (Barcroft, 1946), it is now believed that a competition for circulating anabolites develops between cells, which bears unequally on those of different types. Should a comparable contest for nutrients develop in neoplasia, the processes associated with the multiplication of normal cells might be amongst the first to reflect a deterioration in the metabolic state of the host. It this were so, the study of changes in mitotic counts of normal cells in tumour-bearing mice whose diets were fortified with various supplements might disclose the nature of some anabolite that is required for the essential processes inherent in cell division.

SUMMARY.

1. A reduction in the mitotic activity of epidermal cells underlying normal "physiological regeneration" in the skin has been observed in mice bearing a transplantable tumour at a distant site.

2. This reduction in mitosis becomes significant when the tumour reaches a weight equivalent to about 10 per cent of the host's body weight. It is found in mice which have undergone no wasting and which show no signs of impairment in their general condition.

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EPIDERMAL MITOTIC ACTIVITY AND CARBOHYDRATE METABOLISM IN THE TUMOUR-BEARING MOUSE.

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Received for publication August 8, 1952.

In a previous paper Laws and Wright (1952) have shown that the growth of an implanted sarcoma in the Bagg albino strain of mouse is associated with a reduction in the mitotic activity of the normal epidermis of the pinna. This fall was apparent when the tumour reached about 10 per cent of the body weight of the host. From evidence presented there, it seems unlikely that the decline can be attributed to any nutritional inadequacy or to any lack of animal protein in the diet consumed. In view of Bullough's (1948, 1949, 1950, 1952) accounts of the effects of alterations in the amount of available carbohydrate upon the diurnal variation of mitotic activity in the epidermis of normal mice, it seemed desirable to obtain comparable observations on our tumour-bearing animals. It was hoped, moreover, that such a study might throw some further light on the metabolic processes involved in mitosis.

MATERIALS AND METHODS.

Most of the materials and methods used in this study have already been described (Laws, 1952 ; Laws and Wright, 1952) ; only a summary will therefore be given here.

Albino mice of the Bagg substrain C, inbred by brother-sister matings, were used. They were kept on Rowett Research Institute stock cube diet ; food and water were freely available. The tumour was the Sarcoma 37 of the Imperial Cancer Research Fund. Epidermal samples were obtained from the pinna, and after their separation from the underlying connective tissues they were stained by Feulgen's method. Mitotic activity was estimated by counting the division figures seen in 100 microscopic fields under a magnification of 540 diameters.

Blood-sugar determinations were made on tail vein blood by the method of Haslewood and Strookman (1939). The starch suspensions injected subcutaneously contained 20 mg. in 0·5 ml. of saline.

RESULTS.

1. *Blood-sugar values in control and tumour-bearing mice.*

In experiments previously recorded (Laws and Wright, 1952), a significant difference was observed between the mitotic activities in the epidermis of the pinna in control and tumour-bearing mice. In order to compare the blood-sugar values in these two groups of animals, samples of blood were obtained at the same time of day (10 a.m.) as the skin biopsies. Two such experiments were

performed (July, 1950, and April, 1951), and since the mean values found for the two sets of control mice differed somewhat—possibly from some seasonal influence—the results are recorded separately in Table I. It will be seen that in neither experiment was there any significant difference (as judged by Fisher's test for differences of means of small samples) between the mean blood-sugar values in the control and tumour-bearing mice.

TABLE I.—*Mean Blood-Sugar Values in Groups of Control and Tumour-Bearing Mice.*

Experiment number.	Time of year.	Mean blood-sugar values (mg./100 ml.).		Probability that the observed difference between groups is due to chance. "P".
		Control.	Tumour-bearing.	
1	July	105·0 (20)*	99·5 (18)	>0·5
2	April	94·2 (5)	79·6 (7)	>0·1

* Figures in parentheses show numbers of animals used.

2. *Variations in blood-sugar values and mitotic activity in control and tumour-bearing mice between 10 a.m. and noon.*

In Bullough's (1949) experiments, in which an alteration in the epidermal mitotic activity in normal mice appeared to be correlated with changes in blood-sugar values, considerable changes were observed for both variables over the 2-hour period from 10 a.m. to noon. It was decided, therefore, to make similar comparisons for our control and tumour-bearing mice. The results are given in Table II.

TABLE II.—*Mean Blood-Sugar Values and Epidermal Mitosis Counts in Control and Tumour-Bearing Mice at 10 a.m. and at Noon.*

Groups of animals.	Mean blood-sugar values (mg./100 ml.).			Mean mitosis counts (mitoses per 100 fields).		
	10 a.m.	Noon.	"P."*	10 a.m.	Noon.	"P."
Control (5)	94·2	95·0	>0·9	17·8	9·0	>0·1
Tumour-bearing (7)	79·6	84·4	>0·3	3·4	6·3	>0·6

* "P" shows the probability of the observed difference between the findings for each group at 10 a.m. and at noon, being due to chance.

From Table II it can be seen that in the control mice the mitotic activity declined markedly between 10 a.m. and noon. Although the change recorded here has only a moderate level of significance, other experiments with the same strain of mice (Laws, 1952) have confirmed that this morning decline is a real one. The blood-sugar values both of these control mice and of those bearing tumours, however, showed no significant concomitant change; nor was there any significant alteration in the mitotic activity of the epidermis in the latter group of animals.

3. *Correlations between tumour size, mitotic activity and blood-sugar value.*

Since the growth of the tumour seemed to affect detrimentally the mitotic activity of the epidermis, coefficients of correlation were calculated between proportionate tumour size, mitotic activity and blood-sugar value for a group

of mice whose tumours varied between 6 and 20 per cent of their body weight. The values of these coefficients are given in Table III.

TABLE III.—*Coefficients of Correlation between Tumour Size, Mitotic Frequency and Blood-Sugar Values in a Group of Tumour-Bearing Mice.*

(*a*) Tumour size and blood-sugar value (19) . .	$-0{\cdot}10 \pm 0{\cdot}24$
(*b*) Tumour size and mitotic frequency (21) . .	$-0{\cdot}46 \pm 0{\cdot}22$
(*c*) Blood-sugar value and mitotic frequency (19) .	$0{\cdot}24 \pm 0{\cdot}24$

The only coefficient which possessed any significance, and that of only a moderate order, was the one between the relative weight of the tumour and the mitotic activity of the epidermis.

4. *The effect of injections of starch suspensions on the mitotic activity in the epidermis of tumour-bearing mice.*

A possibility remained that some local deficit of carbohydrate in the epidermal cells, which was not reflected in the blood-sugar value, might diminish their rate of mitosis. It was therefore decided to try the effect on mitotic activity in tumour-bearing mice of a subcutaneous injection of a suspension of starch, a procedure which had been found by Bullough (1949) to prevent the fall in mitosis that ordinarily takes place in the epidermis of control normal mice between 10 a.m. and noon. For purposes of control a comparable series of tumour-bearing mice received an injection of saline only. The results of these injections at 10 a.m. on the blood-sugar values and mitosis counts at noon are shown in Table IV.

TABLE IV.—*The Effect of Injections of Saline and Starch Suspensions at 10 a.m. on the Blood-Sugar Values and Epidermal Mitotic Frequency in Tumour-Bearing Mice at Noon 2 Hours later.*

Material injected.	Samples taken at noon. Mean blood-sugar values (mg./100 ml.).	"P."*	Mean mitoses counts (mitoses per 100 fields).	"P."
Saline (12) . . .	88·7	>0·1	8·8	>0·9
Starch suspension (11) .	81·0		8·5	

* "P" shows the probability of the observed difference between the saline and the starch suspension groups, being due to chance.

From Table IV it can be seen that at the end of 2 hours neither the mean blood-sugar value nor the mitotic activity of the epidermal cells was significantly affected by the injection of the starch.

DISCUSSION.

In his recent review of the metabolism of mitosis, Bullough (1952) has laid stress upon the dependence of the process on the adequacy of carbohydrate supplies to the tissues. He has further suggested that the well-known diurnal rhythm of mitosis in the epidermis of the mouse may result from phasic changes in the availability of these substances to the growing cells. For neither of these beliefs do the findings of the experiments described here and elsewhere (Laws,

1952) afford any support. It is well recognized that a sharp fall in the mitosis rate of the epidermis of normal mice takes place between 10 a.m. and noon, yet in the present experiments on a group of mice whose mitosis rate showed the typical fall, the mean blood-sugar during that period remained unchanged.

Since the main purpose of the present work was to explore the possibility that alterations in blood-sugar levels might account for the persistent depression of mitosis observed by Laws and Wright (1952) in the epidermis of tumour-bearing mice, the degree of correlation between these two variables was sought for a group of mice that presented a wide range of proportionate tumour size. Though a coefficient of moderate significance was found between tumour size and frequency of epidermal mitosis, those between tumour size and blood-sugar value and between blood-sugar value and frequency of mitosis were both without significance. It seems unlikely, therefore, that the depression in mitosis that develops progressively in the epidermis of the pinna of tumour-bearing mice can be attributed to any inadequacy in the supply of carbohydrate to these cells.

SUMMARY.

1. No significant difference was found between the mean values for the blood sugar in groups of normal control and of tumour-bearing mice.

2. The sharp decline in the frequency of mitosis in the epidermal cells of the pinna that takes place typically in normal mice between 10 a.m. and noon is not associated with any concomitant change in the blood sugar.

3. In a series of mice which bore tumours varying from 6 to 20 per cent of their body weights, there was no significant correlation between the blood sugar value and the epidermal mitotic activity.

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HORMONALLY INDUCED EPITHELIAL HYPERPLASIA IN THE GOLDFISH (*CARRASIUS AURATUS*).

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Received for publication May 29, 1952.

IT is well known among goldfish breeders that the male goldfish develops "tubercles" on the gill plates and pectoral fins during the breeding season and that these changes are not seen in the female. Histological examination of the fin tubercles shows them to be indistinguishable from ordinary papillomata (Fig. 1, 2). In view of this it seemed worth while to see if a similar papillomatous hyperplasia could be experimentally induced by testosterone, as this might provide a possible field for the induction of chemical carcinogenesis, for although numerous instances of a wide variety of naturally-occurring tumours have been recorded in fish (Lucké and Schlumberger, 1949), there is no convincing record of an experimentally-induced tumour (Lucké, 1951, personal communication). Further, during the last two years numerous attempts have been made by us to induce tumours in tropical and cold-water aquarium fish using 9 : 10, dimethyl 1 : 2 benzanthracene and methylchloanthrene without any success.

Apart from the epithelial hyperplasia seen in all types of male goldfish during the breeding season, there occurs in a rather rare variety of goldfish (Bramble Head) (Fig. 3, 4) an extreme degree of epithelial hyperplasia covering the gill plates, which is, presumably, genetically determined.

METHODS.

Experiment A.—Six common goldfish (4 males and 2 females) approximately one year old, of mean length 2½ in. from snout to root of caudal fin, received a total of 4 mg. each of testosterone propionate (Perandren), intraperitoneally, in divided weekly doses over a period of 4 weeks. The fish were inspected every day and at the end of a fortnight (17.vii.51) were killed.

Experiment B.—A further group of 10 adult goldfish (6 males and 4 females) of mean length 5½ in. from snout to root of caudal fin, received an intraperitoneal implant of a 10 mg. pellet of testosterone on 28.ix.51 through a stab incision, under ether anaesthesia. The fish were observed daily and were killed on 2.xi.51.

Experiment C.—Twenty-four goldfish, of mean length 2½ in. from snout to root of caudal fin, and about one year old, were divided into two equal groups, an experimental and a control. The experimental animals (8 males and 4 females) were given 10 mg. testosterone intraperitoneal implants, under ether anaesthesia, while the control animals underwent a sham operation. Each group was kept in separate tanks under identical conditions of crowding, feeding, light and temperature (56° F.). The experiment was commenced on 29.i.52, and all fish were killed on 18.ii.52 and their sex determined.

Paraffin sections, stained H. & E., were cut from gills, pectoral fins and gonads of all fish.

RESULTS.

Macroscopically.

At the commencement of all experiments none of the fish showed any tubercles on either the gill plates or the pectoral fins.

Experiment A.—In the first group the initial tubercles were observed on the gill plates of one of the males 11 days after commencement of treatment, and all except one female showed varying degrees of change on the gill plates within 6 weeks. However, only 3 fish (including one female) showed minute tubercles along the anterior margins of the pectoral fins.

Experiment B.—In this group the initial tubercles were seen on the gill plates of 2 males 13 days after commencement of treatment. Three weeks after implantation of the hormone 7 fish were showing gill tubercles (Fig. 5) ; of these 7, only 5 showed changes on the pectoral fins ; the 3 that showed no change were all females.

Experiment C.—Six days after implantation tubercles appeared on one of the experimental fish. By the 20th day of the experiment 11 of the 12 fish showed tubercles. The one not showing tubercles was a female. None of the control fish showed any tubercles on the gills or pectoral fins during this period.

Microscopically.

Gills.—The hormonally-induced and the naturally-occurring tubercles are seen to consist of a mass of squamous cells forming a conical projection covered with a cap of keratin (Fig. 6, 7). In both these cases a change was observed in the character of the epithelium at this site from a mucus-secreting transitional epithelium to squamous.

Pectoral fins.—Both the naturally-occurring and hormonally-induced tubercles (Fig. 8, 1, 2) on the pectoral fins show a similar type of hyperplasia with a change to a squamous epithelium and a papillomatous structure.

DISCUSSION.

The results of our experiments show that an epithelial hyperplasia can be rapidly produced in immature and adult goldfish of either sex by testosterone, although the effect, as expected, is more readily obtained in male fish. This change is similar to that seen in the male goldfish during the breeding season. It is worthy of note that there is also a change in the character of the epithelium from a transitional mucus-secreting variety to a squamous keratinising epithelium. While the gill tubercles appear to regress completely after the breeding season the pectoral papillomata do not regress completely, leaving a residual nodule which grows progressively larger with each breeding season.

It is interesting to note that in the Bramble Head variety, though the tendency to hyperplasia is genetically transmitted to the offspring, there is probably also a hormonal factor involved as this change is seen almost entirely in the male progeny (personal communication from W. J. Page, editor of 'Water Life', 1951), and further it should be noted that the change to squamous epithelium from mucous transition

epithelium seen in the breeding tubercles is absent in the Bramble Head and there is a myxomatous degeneration in the stroma (Fig. 4).

It is known that testosterone is a mitotic stimulant. Bullough (1950) has shown that when mice are treated with testosterone there is increased mitotic activity and thickening of the epidermis. The results of our experiments show that an epithelial hyperplasia can be produced by the mammalian hormone, testosterone, in a cold-blooded animal such as the fish.

It is possible that a hyperplastic epithelium such as that produced by testosterone in the goldfish may provide a suitable site for the induction of a neoplasm by chemical carcinogenesis, as it is well known that hyperplastic states are often a precursor to neoplasia.

SUMMARY.

(1) Epithelial hyperplasia of the gills and pectoral fins was seen in immature and adult goldfish after treatment with testosterone.

(2) This change was seen in all the male goldfish, but 50 per cent of the female fish did not respond.

(3) The hyperplastic areas have a papillomatous structure on the fins and mammillary appearance on the gill plates. Associated with this there is a change in the nature of the epithelium from mucus-secreting transition to squamous.

We would like to thank Professor H. N. Green for valuable advice and criticism. We are indebted to Mr. C. F. Whitehead for the specimen of the Bramble Head goldfish and to Mr. A. W. Collins, F.I.M.L.T., for the photographs. Testosterone implants were kindly supplied by Dr. W. J. Tindall of Organon Laboratories Ltd.

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EXPLANATION OF PLATES.

Fig. 1.—Naturally-occurring breeding tubercle (papilloma) on the pectoral fin of an adult male goldfish approximately 5½ in. long. H. & E. × 7.

Fig. 2.—High-power view from Fig. 1.

Fig. 3.—Bramble Head goldfish showing luxurious growth covering head and gill plates. Note translucent appearance of tumour produced by myxomatous change in stroma. × 1.

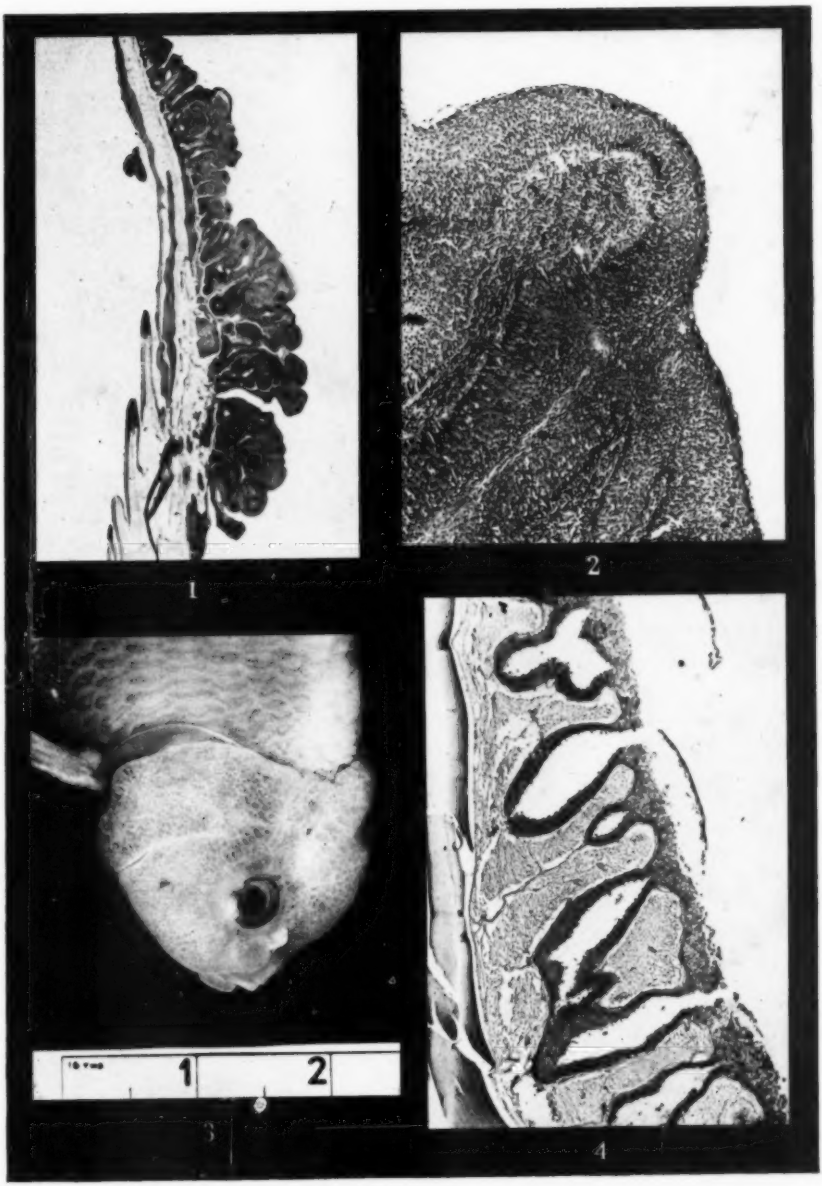
Fig. 4.—Section through growth on Bramble Head goldfish showing a papillomatous hyperplasia of the mucus-secreting epithelium and a myxomatous change in stroma. H. & E. × 21.

Fig. 5.—Hormonally induced tubercles on gill plates and on anterior edge of pectoral fin. × 1.

Fig. 6.—Section through hormonally induced gill-plate tubercle. Note change from mucus-secreting transitional epithelium to a squamous type. H. & E. × 90.

Fig. 7.—Naturally-occurring breeding tubercle on gill plate. H. & E. × 90.

Fig. 8.—Hormonally-induced hyperplasia on anterior edge of pectoral fin showing an early papillomatous structure. H. & E. × 50.



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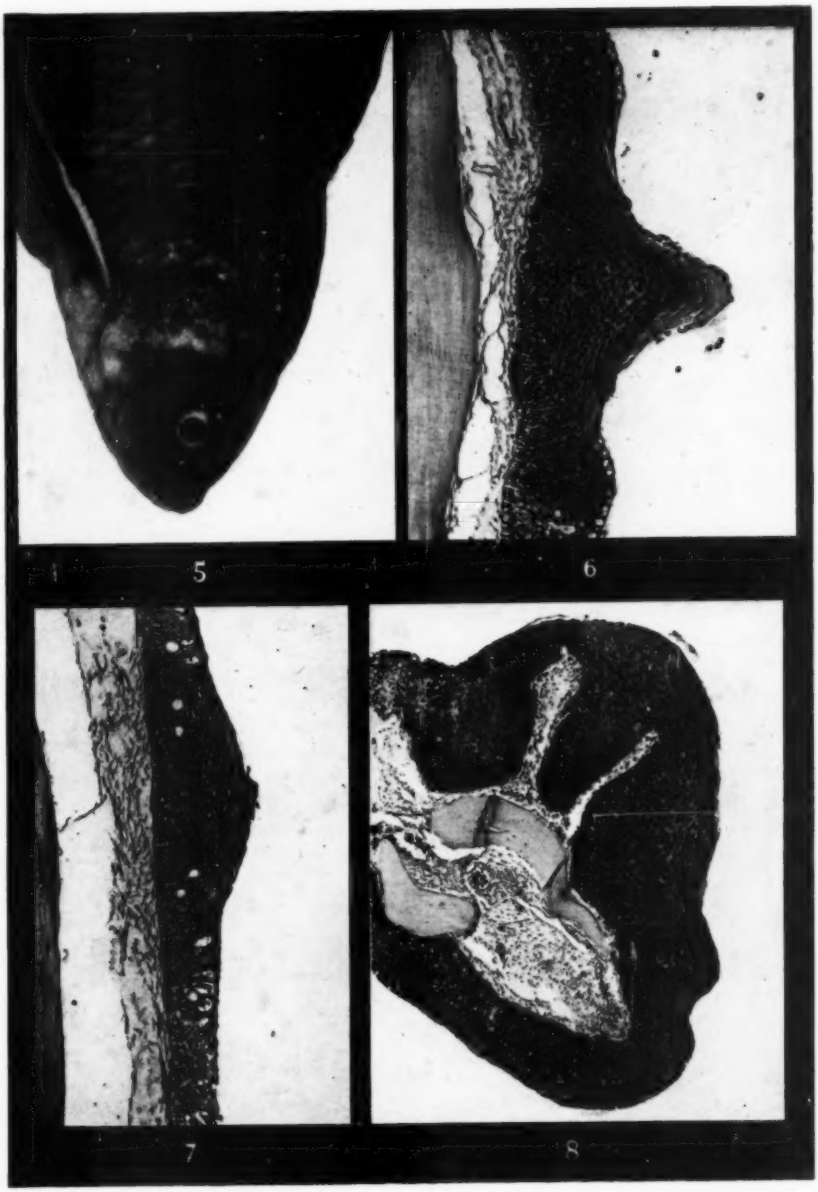

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BEHAVIOUR OF THE MAMMARY TUMOUR INDUCING AGENT IN A TRANSPLANTABLE MAMMARY TUMOUR IN MICE.

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Received for publication June 7, 1952.

In experiments previously reported (Dmochowski, 1949) the mammary tumour inducing agent was demonstrated in the cells of a 42nd serial transplant of a breast tumour, originally induced in a C57 black low-breast-cancer-strain mouse after foster nursing by RIII high-breast-cancer-strain female. However, it was not possible at that stage to state what part, if any, is played by the agent in the propagation of the transplanted tumour cells. It was then pointed out that in order to throw some light on the question as to whether the presence of the mammary tumour inducing agent is a permanent feature of the transplanted tumour cells, they will have to be tested again for the agent after a series of passages through C57 black low-breast-cancer-strain mice, known to be free of the agent. Serial transplantation of this tumour, designated as C57× breast tumour, in C57 black mice presented no difficulty, as it had previously been shown (Bittner, 1947 ; Dmochowski, 1949) that transplantability of a breast tumour induced in low-cancer-strain mice by foster-nursing by high-cancer-strain females is not connected with the presence of the agent in the hosts (C57 black mice), and the C57× transplanted tumour grew equally well in females and males of the C57 black strain (Dmochowski, 1949). Further, the rate of growth of the C57× breast tumour was from the beginning comparatively fast, and remained at an average of 21 days throughout all the serial transplants. This made it possible to carry out experiments in order to ascertain whether the mammary tumour inducing agent could be detected in this breast tumour after 86 serial transplants in agent-free C57 black low-breast-cancer-strain mice.

METHODS.

C57 mammary tumour tissue of the 86th serial transplant, desiccated in the same way as tumour tissue of the 42nd transplant (Dmochowski, 1946, 1949), was used as the source of material. Before use, the desiccated tumour tissue of the 86th transplant was resuspended in distilled water in a proportion of 1:10, and injected intraperitoneally every second day in 1·0 c.c. quantities into the test mice. The intraperitoneal route was selected in preference to the subcutaneous employed in the test of the 42nd transplant because of its greater effectiveness. Each injection contained an amount of dried tissue equal to 0·1 g. of fresh mammary tumour tissue. Each test mouse received 10 injections, corresponding to a total of 1·0 g. of fresh mammary tumour tissue. RIII high-cancer-strain spontaneous tumour tissue, dried, resuspended in distilled water in a proportion

of 1:10, was injected intraperitoneally into control mice. Each control animal received one injection containing material corresponding to 0·1 g. of fresh tumour tissue.

(C57 × A) F_1 hybrid mice, born and raised in the laboratory, were employed as test mice. These mice were used in preference to (C57 × RIII) F_1 hybrid mice, because they were found to be equally susceptible in tests with material containing the agent. Moreover when used as controls they developed a lower incidence of mammary tumours (less than 3 per cent) following the removal of the first three litters within 24 hours after birth. The (C57 × A) F_1 hybrid females, when 3 to 4 weeks old, were divided into experimental and control mice. Both experimental and control hybrid females were forcibly bred by removing the first three litters within 24 hours after they were born, and then allowed to breed in a normal way.

RESULTS.

The results of the experiment are shown in Table I.

As can be seen from Table I, no tumours developed in any of the 17 (C57 × A) F_1 hybrid mice given repeated injections of dried tissue of 86th transplant of the C57 × mammary tumour, while the control mice, given a single injection of material known to contain the agent, developed a comparatively high incidence of mammary tumours. Thus, the presence of the mammary tumour agent in the 86th transplant of the C57 × mammary tumour could not be demonstrated by the same method which had previously revealed the agent in the 42nd transplant, and now again in material known to harbour the agent, when tested in litter-mate control mice.

DISCUSSION.

The mammary tumour inducing agent was found to be present in mammary tumour cells following ten passages of a mammary tumour which developed in a C57 black low-cancer-strain female after foster-nursing by a high-cancer-strain female (Bittner, 1948), after 42 passages of a similarly induced tumour (Dmochowski, 1949), and also in high-cancer-strain tumour cells after 31 passages in fertile eggs (Armstrong and Ham, 1950). The presence of the agent in mammary tumour cells after 42 transplantations in C57 black low-cancer-strain mice characterised by low susceptibility to mammary cancer and lack of the agent again raised the question what part is played by the mammary tumour inducing agent in the propagation of the transplanted breast tumour cells, and whether the cells will continue to multiply in the same way should the agent disappear from the cells. Bittner, Evans and Green (1945) reported the presence of the agent after 10 serial passages of high-cancer-strain tumour cells in agent-free but susceptible mice, and concluded that the agent is continuously produced in the transplanted tumour cells.

In an attempt to ascertain whether the presence of the agent is a permanent feature of the transplanted cells, the C57 × transplantable mammary tumour was again tested for the presence of the agent after 86 serial transplants in C57 black low-cancer-strain mice. The method of preparation of the tissue for testing and the test itself were the same as those employed for the 42nd transplant of the tumour in which the agent was found to be present. The test failed, however,

TABLE I.—*Administration of Dried C57× Mammary Tumour Tissue of the 86th Transplantation and of Dried RIII Strain Spontaneous Mammary Tumour Tissue to (C57×A) F_1 Hybrid Female Mice.*

Number of mice injected.	Age of mice when first injected (weeks).	Origin of tumour tissue.	Number of injections.	Total of fresh tumour tissue injected (g.).	Number of mice dying before earliest tumour appearance.	Numerators = Number of tumours appearing monthly. Denominators = Number of mice dying monthly.												Number of mice with tumours (%).	Average tumour age (months).	Average age of mice dying without tumours (months).
						7	9	10	12	13	14	15	16	17	18	19	20			
18	3–4	C57×	10	1·0	1	–	–	–	$\frac{0}{1}$	–	$\frac{0}{1}$	$\frac{0}{4}$	$\frac{0}{4}$	$\frac{0}{2}$	$\frac{0}{2}$	$\frac{0}{3}$	–	—	—	16·3
12	3–4	RIII	1	0·1	3	$\frac{1}{0}$	$\frac{1}{0}$	$\frac{2}{0}$	$\frac{1}{0}$	$\frac{1}{1}$	$\frac{0}{1}$	–	–	–	–	–	$\frac{0}{1}$	6 out of 9 (66·6)	10·1	15·7

to reveal the agent in the cells of the 86th tumour transplant. It must be mentioned that the histological appearance of this tumour has remained unaltered, in spite of the many serial passages and the apparent absence of the agent. It is still the same, that of a typical mammary adenocarcinoma, although the tumour is now in its 118th transplant.

A similar observation on the recovery and disappearance of the rabbit papilloma virus from carcinomata which originated from papilloma cells has been reported by Smith, Kidd and Rous (1947). They succeeded in the recovery of small amounts of papilloma virus from the first and second transplant of carcinoma cells in new-born rabbits, but failed to recover the virus from tumours of the third and fourth passage. The same authors reported positive neutralisation and complement-fixation tests between papilloma virus and sera of adult rabbits in which a carcinoma originally derived from a virus papilloma was transplanted for 22 successive passages. Sera of rabbits carrying the 46th and 50th tumour transplants failed to give a positive complement-fixation test with the papilloma virus or to neutralise it. They concluded that the virus was no longer present in the tumour, although the morphology of the tumour remained unaltered. Attempts to reinfect the tumours by placing them in a virus suspension or injecting papilloma virus intravenously into rabbits bearing transplants of the carcinoma yielded dubious results.

Serological tests in mice similar to those carried out in rabbits by Smith, Kidd and Rous (1947) in order to ascertain the presence of a latent or masked mammary tumour inducing agent in cells of the 86th transplant could not be of any help in this respect. No antibody could be detected by means of complement-fixation tests with ultracentrifugal deposits of high-cancer-strain tumour extracts in sera of high- and low-cancer-strain mice, whether normal, bearing different transplantable mammary tumours or even immunised with material containing the agent (Dmochowski and Passey, 1951). No attempts were made to test the cells of the 86th transplant C57 × mammary tumour by grafting the tumour tissue into susceptible mice in view of the previous observations by various authors. Thus transplantation of mammary tumour tissue with the agent into susceptible mice has either failed to transfer the agent (Andervont, 1949), or has induced only a low incidence of tumours (Hummel and Little, 1949). It has also been found to be a poor source of the agent (Barnum, Ball and Bittner, 1947). It was therefore considered an unsatisfactory method for testing the transplantable C57 × tumour for the presence of the agent. Reinfection of the tumour tissue with the mammary tumour agent was not carried out because of the observations of Smith, Kidd and Rous (1947), although it is quite possible that it would have yielded unequivocal results. In connection with the negative result obtained with the 86th transplant, another test for the presence of the agent in cells of the 101st tumour transplant was carried out. So far no tumours have been observed in any of the test mice, but it is too early to draw a conclusion, as all the mice are still alive, having lived now for 11 months. Bittner (1952, personal communication) has found the agent present in the 15th transplant of a C3H mammary tumour in agent-free C3H mice, while in the cells of the 7th transplant of the same tumour it was apparently difficult to detect. It is not known whether a similar situation may arise with the C57 × transplantable mammary tumour, although the agent could not be detected in the 86th serial passage by the same means which revealed its presence in the 42nd transplant. The results show that the

tumour cells continue to multiply although the agent has apparently disappeared from the cells.

In search of a possible explanation of the " disappearance " of the mammary tumour inducing agent from the cells of a transplantable mammary tumour several interpretations may be put forward. The agent or Bittner virus, as it is described by some authors, may have been altered in some unknown way, possibly by mutation. It may have become closely associated with one of the particulate elements of the tumour cells. This close integration of the agent with the tumour cells may have produced a state in which it was unable to survive the death of the tumour cells during extraction procedures preparatory to the test. The virus or agent may indeed have disappeared after many serial passages through low-breast-cancer-strain mice, or it may have become " toothless ", as postulated by Andrewes (1934), that is, an agent which may cause proliferation but will not infect new cells. It could be argued that a failure to detect a viral agent in a tumour by infectivity tests need not necessarily mean its absence from the tumour, as it may be present in very small amounts too difficult to detect, although it is difficult to see how this could be proved experimentally. It is possible that the observed disappearance of the mammary tumour agent may somehow be connected with the mode of its multiplication, although at present it is difficult to state how it is connected.

SUMMARY.

The mammary tumour inducing agent or Bittner virus has not been detected in the cells of the 86th transplant of C57 × mammary tumour, originally induced in a C57 black low-cancer-strain mouse by foster-nursing by high-cancer-strain female, although the method employed for testing was the same as that which revealed the presence of the agent in the cells of the same tumour while in its 42nd transplant in agent-free C57 black strain mice. Several interpretations of this observation are discussed. The results indicate that the tumour cells continue to multiply although the agent responsible for their development cannot be detected.

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THE EFFECT ON THE ROUS NO. 1 FOWL SARCOMA VIRUS OF BERKEFELD FILTRATION WITH AND WITHOUT PREVIOUS REPEATED FREEZING AND THAWING.*

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Received for publication July 17, 1952.

It has been reported in the past that Berkefeld filtrates made from Rous No. 1 fowl sarcoma material which had been subjected to repeated applications of cold possessed a greater tumour-producing activity than those prepared from similar material which had not been subjected to this treatment (Selbie and McIntosh, 1939). In explanation of this finding Selbie and McIntosh (1939) suggested that the freezing broke up the tissue fragments, and thus allowed a greater liberation of virus than would otherwise have been possible.

In the course of some recent quantitative investigations into the effects of cold and desiccation on the Rous No. 1 fowl sarcoma (Epstein, 1951) it was found that when Rous tumour material was repeatedly frozen and thawed and then extracted, no such greater liberation of virus took place than where no treatment was applied. Virus suspensions made from the treated material had less tumour-producing activity than those made from similar untreated samples; thus the suggestion made by Selbie and McIntosh (1939) was not confirmed.

However, although no increased liberation of virus from Rous material subjected to repeated freezing and thawing could be demonstrated to account for the increased tumour-producing activity of Berkefeld filtrates made from such material reported by Selbie and McIntosh (1939), there remained another possible explanation of their finding. The increased tumour-producing activity of Berkefeld filtrates prepared from Rous tumour subjected to repeated applications of cold might have been due to some action of the cold on the virus whereby its physical state was altered in such a way as to allow it to pass the filter with greater facility than where no treatment was applied. It was therefore decided to investigate the effect of repeated applications of cold on the filterability of the Rous virus as distinct from its extractability.

As a preliminary to this it was considered necessary to examine the effect of Berkefeld filtration on suspensions of the Rous virus. For although it has long been known that the procedure may render highly active Rous virus preparations inert (Rous and Murphy, 1914), and although this has, since it was first reported, often been taken for granted (Cramer and Foulds, 1930), few experimental investigations of the phenomenon have been undertaken. That particles in suspension became adsorbed on to Berkefeld filters during filtration has been known for some time and the influence of pH upon this has been demonstrated (Mudd, 1922;

* This communication formed one part of a thesis approved by the University of London for the degree of Ph.D.

Kramer, 1927). However, no work of this kind has been reported with regard to the Rous virus. The chromatographic study of Riley (1950) in which adsorption of the Rous virus was shown to take place on to a column of diatomaceous earth suggested that adsorption would also take place when this virus was passed through Berkefeld filters which are likewise composed of diatomaceous earth.

In the present work an attempt has been made to assess the decrease in the tumour-producing activity of suspensions of the Rous No. 1 fowl sarcoma virus which Berkefeld filtration brings about. Experiments are also reported which were designed to show whether or not this decrease was due to adsorption of the virus on to the Berkefeld candle during filtration, and in addition, an investigation has been carried out to find whether repeated freezing and thawing affects the filterability of the Rous virus. A titration technique has been used.

MATERIALS AND METHODS.

Tumour.—The Rous No. 1 fowl sarcoma used was derived from that introduced into the Bland-Sutton Institute in 1925, and maintained by passage since then.

Animals.—Pedigreed Brown Leghorn fowls from the Institute of Animal Genetics, Edinburgh, have been used in the experiments. They were between 7 and 17 weeks old when inoculated, and belonged to three susceptible inbred lines developed at Edinburgh, or crosses derived from them. The lines and crosses used were Intensity, Breeding, Non-moult, Breeding hen × Intensity cock, Breeding hen × Non-moult cock, and Intensity hen × Breeding cock, and have been described by Greenwood, Blyth and Carr (1948); both the age at which the birds were inoculated and the line or cross to which they belonged were determined by the exigencies of supply.

Preparation of tumour material for the experiments.

For each experiment a bird with a large actively growing sarcoma of anything from 9 to 18 days' growth from the date of inoculation was selected as a source of tumour material and killed by wringing of the neck. The tumour was removed, cut small with scissors, and disintegrated in a mechanical blender; the method has been described in detail in a previous paper (Epstein and Cook, 1951). When prepared, the disintegrated tumour was loaded into a Record syringe for easy manipulation.

Techniques used in the experiments.

Repeated freezing and thawing.—Where disintegrated tumour was to be treated in this way it was loaded into ampoules from a Record syringe, shell-frozen, and at once thawed. The process of freezing and thawing was rapidly repeated five times, after which the material was ready for use. A detailed account of the method has previously been given elsewhere (Epstein, 1951).

Berkefeld filtration.—Material to be filtered in this way was passed through a Berkefeld V candle (German laboratory type 12, $2\frac{1}{2} \times \frac{5}{8}$ in., supplied by Baird & Tatlock, Ltd., London) using a negative pressure of 380–510 mm. Hg. obtained by means of a water pump. Filtration was continued until the filter candle was quite empty, as shown by air bubbles being sucked through after the filtered fluid. After use each filter candle was tested by passing through it 80 c.c. of an

overnight broth culture of *Staph. aureus* and *Chr. prodigiosum* diluted 1 in 16 in broth. Of the filtrate obtained in this way 1 c.c. was incubated in a tube of broth at 37° C. for 18 hours and was examined after that period for the presence of the test organisms. The results of these tests are shown with the corresponding experimental results in Tables I, II and III.

Preparations used for inoculation.

All samples of disintegrated tumour, both those left untreated and those subjected to repeated freezing and thawing, were made in each experiment into some or all of the following preparations :

Virus suspension.—This consisted of a 10 per cent. vol./vol. suspension of disintegrated tumour in physiological saline from which the cells were separated off by centrifuging in an angle centrifuge at 3500 r.p.m. for 15 minutes, decanting the supernatant fluid obtained, and centrifuging it further in an horizontal centrifuge at 6000 r.p.m. for another 15 minutes. The resulting clear supernatant fluid was decanted and used here as the virus suspension.

Berkefeld filtrate.—This was prepared by passing virus suspension through a Berkefeld filter in the manner already described above.

Saline wash.—Physiological saline was passed through a Berkefeld filter after it had been used and was collected in a fresh unused filter flask ; the volume of the saline was kept the same as that of the original material for which the filter had been used.

Experimental procedure.

Experiments 1, 2 *and* 3 (effect of filtration).—Disintegrated Rous tumour was prepared and from it a virus suspension was made as has been described. A 1 c.c. sample of the virus suspension was taken and diluted in serial tenfold steps with physiological saline, a portion of each dilution being kept ready for inoculation ; the rest of the virus suspension was passed through a Berkefeld filter. A sample was taken from the resulting filtrate, part of it being set aside ready for inoculation and part being used to make dilutions in serial tenfold steps with saline ; a portion of each dilution was kept for inoculation.

Experiments 4 *and* 5 (recovery of virus from filter).—These experiments were performed in their early part as a repetition of the foregoing in which samples of a virus suspension and the Berkefeld filtrate derived from it were collected and diluted in serial tenfold steps with saline for inoculation. In addition, however, a saline wash of the filter was prepared ; a sample was taken from the saline wash, part being set aside undiluted for inoculation and part being used to make dilutions in serial tenfold steps with saline. A portion of each dilution was kept for inoculation.

Experiments 6, 7, 8 *and* 9 (effect of freezing and thawing on filterability).—Disintegrated tumour was prepared and in each of these experiments was divided into two samples, one being left untreated and the other being subjected to repeated freezing and thawing. The two samples of disintegrated tumour, that left untreated and that subjected to repeated freezing and thawing, were each made into a virus suspension and a Berkefeld filtrate ; all of these preparations were diluted in serial tenfold steps with saline ready for inoculation.

Inoculations.—Inoculations were made into the breast and thigh muscles of

fowl; each inoculum was 0·5 c.c. in volume, and four inoculations of each preparation or dilution of a preparation were made. The scheme used for the distribution of the inoculations amongst the birds has been described elsewhere (Epstein, 1951).

Examination of fowl.—All the birds were examined every week, and all the tumours which developed, including those few which subsequently regressed, were recorded. Every bird which died or which was killed at the conclusion of an experiment was submitted to *post-mortem* examination; histological investigations were carried out upon all material the nature of which could not be diagnosed macroscopically.

Duration of experiments.—The experiments were continued until all the tumour-bearing birds had died, or for a minimum period of nine weeks, after which time the surviving birds were killed.

General considerations.

Inoculations were taken as having caused a tumour in all cases where tumours were palpated at the site of inoculation. or where a tumour was found there at *post-mortem* examination.

Strict aseptic technique was maintained throughout all the experimental procedures. All the experimental procedures were timed. None was of more than 4½ hours' duration up to the moment of completing the inoculations and thus the time in which gradual inactivation of the virus by oxidation (Pirie and Holmes, 1931) could take place was standardised.

RESULTS.

Table I shows that the tumour-producing activity of a Berkefeld filtrate of the Rous sarcoma was in each case less than that of the virus suspension from which it was made; the reduction in this activity which followed the filtration was in one case very considerable (Experiment 3).

TABLE I.—*The Effect of Berkefeld Filtration on the Tumour-producing Activity of Preparations of the Rous Virus.*

Expt. No.	Preparation of disintegrated tumour.	Dilutions of preparation and number of tumours arising from 4 inoculations of each.						Filter test.	
		10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	*prod.*	*Staph.*
1	10% virus suspension	–	–	3	3	0	0	–	–
	Berkefeld filtrate	4	4	3	0	–	–	R	R
2	10% virus suspension	–	–	4	4	1	0	–	–
	Berkefeld filtrate	4	4	3	0	–	–	P	R
3	10% virus suspension	–	4	4	2	1	0	–	–
	Berkefeld filtrate	1+(1)	0	0	0	0	–	R	R

Tumours which regressed shown in brackets.
P = passed. R = retained.

It will be seen from Table II that where physiological saline was passed through a Berkefeld candle immediately after the latter had been used to filter a Rous sarcoma virus suspension, the saline acquired a considerable tumour-producing

activity. In addition, the results shown in Table II confirm those of Table I regarding the reduction in the tumour-producing activity of Rous sarcoma virus suspensions which takes place when these are passed through a Berkefeld filter candle.

TABLE II.—*The Tumour-producing Activity of Saline Passed through Berkefeld Candles Previously used to Filter Preparations of the Rous Virus.*

Expt. No.	Preparation of disintegrated tumour.	Dilutions of preparation and number of tumours arising from 4 inoculations of each.						Filter test.	
		10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	*prod.*	*Staph.*
4	10% virus suspension	–	4	4	0	0	0	–	–
	Berkefeld filtrate	–	4	1	0	0	–	R	R
	Saline wash	4	2	1	0	–	–	–	–
5	10% virus suspension	–	4	4	4	1	0	–	–
	Berkefeld filtrate	–	3	1	0	0	0	R	R
	Saline wash	3	3	2	0	0	–	–	–

Tumours which regressed shown in ( ).
P = passed. R = retained.

From Table III it is evident that the tumour-producing activity of virus suspensions made from disintegrated Rous tumour which had been subjected to

TABLE III.—*The Effect of Repeated Freezing and Thawing on the Tumour-producing Activity of Preparations of the Rous Virus and on their Filterability*

Expt. No.	State of disintegrated tumour.	Preparation.	Dilutions of preparation and number of tumours arising from 4 inoculations of each.						Filter test.	
			10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	*prod.*	*Staph.*
6	Untreated	10% virus suspension	–	4	4	4	2	0	–	–
	Frozen and thawed × 6		–	4	4	3	0	0	–	–
	Untreated	Berkefeld filtrate	3	2	0	0	0	–	R	R
	Frozen and thawed × 6		4	0	0	0	0	–	P	R
7	Untreated	10% virus suspension	–	4	4	4	3	0	–	–
	Frozen and thawed × 6		–	4	4	3	0	0	–	–
	Untreated	Berkefeld filtrate	4	2	0	0	0	–	C	C
	Frozen and thawed × 6		2+(2)	0	0	0	0	–	R	R
8	Untreated	10% virus suspension	–	4	4	4	3	0	–	–
	Frozen and thawed × 6		–	4	4	4	0	0	–	–
	Untreated	Berkefeld filtrate	3	4	2	0	0	–	P	R
	Frozen and thawed × 6		4	4	0	0	0	–	R	R
9	Untreated	10% virus suspension	–	4	3	3	0	0	–	–
	Frozen and thawed × 6		–	3+(1)	3	3	0	0	–	–
	Untreated	Berkefeld filtrate	4	4	2	0	0	–	R	R
	Frozen and thawed × 6		4	4	0	0	0	–	R	R

Tumours which regressed shown in ( ).
P = passed. R = retained. C = test contaminated.

repeated freezing and thawing was usually reduced (Experiments 6, 7 and 8) as compared with those made from untreated material. Where the activity of the preparation made from frozen and thawed disintegrated tumour was not less

than that made from untreated material it was the same (Experiment 9), not greater. Table III also shows that the same order of reduction in tumour-producing activity took place when a virus suspension made from untreated disintegrated tumour was passed through a Berkefeld candle to give a filtrate as when a virus suspension made from repeatedly frozen and thawed material was so filtered. That such a reduction took place confirms the results of Table I.

Also shown in Tables I, II and III are the results of the tests made with each filter after use to determine its power of retaining the organisms of the test cultures. On several occasions the filters failed to retain *Chr. prodigiosum* (Table I, Experiment 2; Table III, Experiments 6 and 8), the smaller of the two test organisms. In one experiment (Table III, Experiment 7) the test was inconclusive owing to accidental contamination.

DISCUSSION.

The results that have been obtained show that where an active virus suspension in physiological saline, prepared from disintegrated Rous tumour, was passed through a Berkefeld V candle, the tumour-producing activity of the resulting filtrate was always less than that of the original virus suspension (Tables I, II and III).

The tumour-producing activity of the virus suspensions is considered to have been due solely to the virus they contained; such suspensions are believed to be largely if not wholly cell-free for reasons which have been considered at length elsewhere (Epstein, 1951). Now, from the results shown in Table II, it is clear that an appreciable amount of the tumour-producing activity lost when virus suspensions were passed through Berkefeld candles could be recovered by washing saline through the latter after use. In the experiments shown in Table II the filters when tested retained in each case both *Chr. prodigiosum* and *Staph. aureus* making it impossible for the lost tumour-producing activity, recovered on washing the filters with saline, to have been due to cells. This recoverability of at least part of the tumour-producing activity lost on filtering Rous virus suspensions gives further support for believing the latter to owe their activity almost wholly to virus.

The reduction in virus content, often very considerable (Table I, Experiment 3), brought about by Berkefeld filtration of Rous virus suspensions appears to be due to adsorption of the virus on to the filter rather than to it being held up by the filter on account of its size. This is considered to be the case since the lost virus was recovered at least in part when saline was washed through the filter (Table II), and since the failure of the filters on three occasions to hold back one of the organisms with which they were tested after use did not affect the amount of virus which came through them as judged by the tumour-producing activity of the filtrates (Tables I and III, Experiments 2, 6 and 8). This view is supported by the chromatographic studies of Riley (1950), in which the Rous virus was adsorbed on to columns of diatomaceous earth—the same material as that of which Berkefeld filters are compased—and was eluted off them by washing with saline, and by work on the adsorption on to Berkefeld filters during filtration of particles other than viruses (Mudd, 1922; Kramer, 1927).

The results of the concluding experiments of the present work (Table III) show that the same order of reduction in tumour-producing activity took place where virus suspensions made from disintegrated tumour treated by repeated

freezing and thawing were passed through a Berkefeld candle to give a filtrate, as where those made from similar untreated disintegrated tumour were so filtered. The application of repeated freezing and thawing to Rous sarcoma material has thus been found not to increase the ability of the virus contained in it to pass Berkefeld filters. The present work has also shown (Table III), in confirmation of that previously reported (Epstein, 1951), that repeated freezing and thawing of mechanically disintegrated Rous tumour material did not release more virus on extraction than where no such treatment was applied. From these two facts it appears that repeated freezing and thawing has no action on the Rous virus likely to make Berkefeld filtrates of treated tumour material, prepared as described here, more active than those made from untreated material. The results shown in Table III demonstrate this clearly ; they are at variance with those reported by Selbie and McIntosh (1939).

In attempting to relate the results of the present work to those of previous investigators with which they do not agree, two possible explanations require consideration :

Firstly, it must be remembered that Selbie and McIntosh carried out their investigations 13 years ago. During that time the Rous virus may possibly have undergone variations affecting its properties which might account for the variations in the experimental results obtained.

Secondly, the method of extracting the Rous virus employed by Selbie and McIntosh (1939) consisted of coarse mincing of the tumour material followed by grinding with sand. Repeated freezing and thawing was then applied to the resulting preparation, and may well have contributed to the achieving of complete disintegration of the tumour and the maximum extraction of virus from it. In the present work, however, disintegration of the tumour material was brought about mechanically before the cold was applied, and would seem to have been so complete that the repeated freezing and thawing could not have any further effect. This is considered to be the most likely explanation of the contradiction between the findings reported by Selbie and McIntosh (1939) and those recorded in this communication.

In consequence of the results of the present work it is thought that where in the future attempts are to be made to demonstrate the presence of viruses in tumours not hitherto known to possess them, Berkefeld filtration, with or without previous repeated freezing and thawing of the material to be filtered, should be avoided. Tumour extracts freed from cells by centrifugation would be more suitable preparations for such work than filtered extracts, being more likely to retain small quantities of a virus than preparations which had been passed through Berkefeld filters with their property of adsorption of virus particles.

CONCLUSIONS.

The present work has shown that the Rous virus becomes adsorbed on to Berkefeld filters during filtration, and that it can be washed out of them, when so adsorbed, with saline. The extent of the loss of virus which always takes place during Berkefeld filtration owing to this adsorption has been demonstrated by the titration methods used, and has been shown, on occasions, to be very great. The alleged increase in tumour-producing activity of Berkefeld filtrates made from Rous sarcoma material subjected to repeated freezing and thawing, as compared with those made from untreated material, has been investigated, and

has not been substantiated with the methods used here. It has been confirmed that repeated freezing and thawing of mechanically disintegrated Rous sarcoma tissue prior to extraction does not cause the liberation of more virus from the cells than takes place when no treatment is applied, and at the same time it has been demonstrated that the process of repeated freezing and thawing does not increase the ability of the Rous virus to pass Berkfeeld filter candles.

From the foregoing it has been concluded that Berkefeld filtration of tumour extracts with or without prior repeated freezing and thawing is not a suitable technique for use in work designed to demonstrate a virus in tumours not as yet known to possess one.

SUMMARY.

Experiments performed to establish the effect of Berkefeld filtration on suspensions of the Rous No. 1 fowl sarcoma virus are described. Also described are experiments in which the effect of repeated freezing and thawing on the filterability of this virus has been investigated. Susceptible inbred Brown Leghorn fowl have been used.

The method used to disintegrate Rous sarcoma tissue mechanically in order to make from it a virus suspension is set forth. The technique used to apply repeated freezing and thawing to disintegrated Rous tumour is indicated, as is the technique used in Berkefeld filtration of suspensions of the virus.

A titration method has been employed in which preparations whose tumour-producing activities were to be compared have been diluted in serial tenfold steps with saline and inoculations made with each dilution.

The results show that when Rous virus suspensions prepared by extracting disintegrated Rous tumour with saline were passed through Berkefeld filters, part of the virus became adsorbed on to the filter. By washing saline through the filters immediately after use much of the adsorbed virus proved recoverable.

The results have confirmed that repeated freezing and thawing of mechanically disintegrated Rous tumour before preparing extracts from it did not allow a greater liberation of the virus than would otherwise have occurred.

It has also been demonstrated that repeated freezing and thawing of disintegrated Rous sarcoma material did not increase the ability of the Rous virus it contained to pass Berkefeld filters when extracted and suspended in saline.

The results are discussed in detail together with the conclusions drawn from them.

The expenses of this investigation were borne by the British Empire Cancer Campaign.

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INHIBITION OF TUMOUR-GROWTH AFTER ORAL AND PARENTERAL TREATMENT WITH NITROGEN MUSTARDS AND TRIETHYLENE MELAMINE.

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Received for publication July 19, 1952.

INJECTIONS of aliphatic nitrogen mustards (Boyland, Clegg, Koller, Rhoden and Warwick, 1948; Stock, 1950) have proved of therapeutic value, but sometimes produce clinically undesirable side effects (Nabarro, 1950). The tris(2-chloroethyl)amine (HN3), for instance, seemed very promising (Wilkinson and Fletcher, 1947; Jiminez Diaz, Merchante, Perianes, Lopez and Puig, 1951), but its use has been discouraged by the great risk of thrombosis at the site of injection (Rhoads, 1946; Nabarro, 1950). Clinical reports on the oral activity of an aromatic nitrogen mustard, NN-di(2-chloroethyl)β naphthylamine, R 48 (Haddow, Kon and Ross, 1948) and of triethylene melamine, T.E.M. (Karnofsky, Burchenal, Armistead, Southam, Benstein, Craver and Rhoads, 1951), therefore, aroused much interest.

Human trials on the hazards of water contamination carried out by the American Army Medical Centre indicated that aliphatic nitrogen mustards might be absorbed after oral administration: HN3 in a total dose of 15 to 18 mg. per man caused transitory nausea, vomiting, anorexia, and the number of W.B.Cs. dropped to 2300 (Jager, Wintrobe, Ginzler and Gordon, 1943). It seemed of interest, therefore, to see if aliphatic nitrogen mustards have an oral activity against tumour-growth comparable to that of R 48 or T.E.M. Tests with these two compounds and with three aliphatic nitrogen mustards, bis(2-chloroethyl)amine (*nor* HN2), bis(2-chloroethyl)methylamine (HN2) and HN3, against transplanted tumour are described in this paper.

TECHNIQUE.

Test tumours.—Subcutaneous transplants of Walker carcinosarcoma and of mouse Sarcoma 37 were used. The Walker tumours were passages of tumour cell suspension in dextrose, prepared by Craigie's mincer technique (Craigie, 1949) and preserved at—70°C. (Craigie, 1949, 1952) at the laboratories of the Imperial Cancer Research Fund. The Walker tumour was transplanted by trocar and Sarcoma 37 by Craigie's mincer technique.

Test animals.—Two age groups of female rats were used as hosts for the Walker tumour:

(*a*) A group of $52 \pm 3{\cdot}6$ g. body weight as recommended by Gye (personal communication); (at this age the regular oestrus cycle has not yet developed and individual variations in the growth rate of the tumours are comparatively small). This group will be referred to as "young" rats.

(*b*) A group of 87 ± 5·6 g. body weight as recommended by Boyland (personal communication). For convenience these rats will be referred to as " adult " rats.

Sarcoma 37 was grown in male mice of 18 to 22 g. body weight.

The animals were fed on Thomson's diet (19·2 per cent protein), the rats receiving carrots in addition. Treatment began one day after implantation of the tumour and was continued on consecutive days, Sunday excepted. The mouse sarcoma was treated daily for 7 days. In the tests against the Walker tumour the total dose was given over 7, 8 or 10 days, and also as a single dose (Boyland, Cleg, Koller, Rhoden and Warwick, 1948; Haddow, Kon and Ross, 1948). Eleven to fifteen animals were used for test.

The water-soluble drugs were dissolved in distilled water immediately before administration and given orally or intraperitoneally. The daily dose was given in 0·4 ml. of distilled water per 100 g. rat. The oral dose was administered by stomach-tube, and immediately afterwards 0·8 ml. of distilled water was passed through the tube to ensure that the whole dose was washed into the stomach. R 48 was given in a single oral dose in arachis oil. Control animals received the solvent by stomach-tube or remained untreated. The Walker tumours and Sarcomata 37 were dissected 14 and 9 days respectively after transplantation, fixed in Bouin's fluid and 70 per cent alcohol, dried between filter-paper, and weighed. The difference in weight between control and treated tumours was analysed statistically. The ratio, mean weight of control tumour divided by mean weight of treated tumours, was used as the measure of the activity of the test compound. Duplicate experiments were carried out at different dates.

RESULTS.

Intraperitoneal treatment.

Given in approximately the maximum tolerated doses, both HN2 and HN3 showed similar suppressive activity against the Walker tumour. The mouse sarcoma, however, appeared to be more responsive to HN3. The Walker tumour was significantly more affected by T.E.M. than by HN2 or HN3; the weight ratio between controls and T.E.M. treated tumours was about 14 times that between controls and tumours injected with the two mustards. When tested against the mouse sarcoma, however, T.E.M. was significantly more active than HN2, but not more active than HN3 (Table I). The maximum tolerated dose of *nor* HN2 was about 10 times that of HN2. When injected in these doses the two compounds had similar activity against the Walker tumour (Table II).

TABLE I.—*Comparative Tumour-inhibitory Activity of Intraperitoneal Treatment with Nitrogen Mustards and Triethylene Melamine.*

Agent.	Daily dose (mg./kg.B.W.).	Total dose (mg./kg.B.W.).	Test tumour.	Effect.	
				Ratio.	Signif. (P.).
HN2 hydrochloride	0·12	1·20	Walker	7·2	<0·01
	0·50	3·50	Sarcoma 37	2·2	0·01
	0·50	3·50		2·3	<0·01
HN3 hydrochloride	0·10	1·00	Walker	7·8	<0·01
	0·50	3·50	Sarcoma 37	7·5	<0·01
Triethylene melamine	0·16	1·60	Walker	104·0	<0·01
	0·25	1·75	Sarcoma 37	4·6	0·01

The monofunctional ethyleneimine in a total dose of 10 mg. per kg. was inactive. NN-bis(2 chloroethyl)p-phenylene diamine hydrochloride in a total dose of 4 mg. per kg. had significant activity (ratio 5·4 ; $P<0·01$).

Oral treatment.

(a) *Test animals fed* ad libitum.—The three aliphatic nitrogen mustards when administered orally also retarded tumour-growth. The maximum tolerated oral dose was about twice the maximum tolerated intraperitoneal dose. Using these doses, both oral and parenteral treatment produced similar effects against the Walker tumour (Table II). The oral activity seemed to be influenced by the distribution of the total dose ; the weight ratio between control and HN2-treated tumours became more significant when the total dose was divided over 7 instead of over 10 daily doses. Boyland, Cleg, Koller, Rhoden and Warwick (1948) found that the parenteral activity of nitrogen mustards increased when the total dose was given as a single injection. When given in a single oral dose the effect of HN3 was negligible, whereas the activity of HN2 and *nor* HN2 seemed to be increased to a certain extent. The oral activity of *nor* HN2 seemed at least equal to that of HN2, and more pronounced than that of HN3, but even the highest weight ratios between control-tumours and those treated with the aliphatic compounds were only about one-quarter of the ratio recorded after treatment with R 48 (Table II). The other aromatic nitrogen mustard tested, NN-bis(2 chloroethyl)p-phenylene diamine hydrochloride, when given as a single oral dose of 4 mg. per kg. was inactive. Larger doses were active but toxic.

T.E.M., though strikingly suppressive after intraperitoneal injection, retarded the growth of the Walker tumour to a negligible extent when given orally. When the maximum tolerated dose of 4 mg. per kg. rat was divided over 10 days, no activity was seen. When the same amount was given in a single dose, tumour-growth was slightly but probably significantly retarded.

TABLE II.—*Tumour inhibitory Activity of Nitrogen Mustards and Triethylene Melamine Administered to Young Rats* ($52 \pm 3·6$ *g.*).

Agent.	Route.	Daily dose (mg./kg.B.W.).	Total dose (mg./kg.B.W.).	Effect.	
				Ratio.	Signif. (P.).
HN2 hydrochloride	Intrap.	0·12	1·2	7·2	<0·01
	Oral	0·28	2·8	5·4	<0·02>0·01
	,,	0·40	2·8	11·9	<0·01
	,,	2·80	2·8	7·6	<0·01
nor HN2 hydrochloride	Intrap.	1·20	12·0	8·1	<0·01
	Oral	2·80	28·0	10·2	<0·01
	,,	28·00	28·0	12·1	<0·01
HN3 hydrochloride	Intrap.	0·10	1·0	7·8	<0·01
	Oral	0·20	2·0	6·5	<0·01
	,,	0·20	2·0	6·6	<0·01
	,,	2·00	2·0	2·3	0·02
	,,	2·00	2·0	2·4	<0·02>0·01
R 48	,,	100·00	100·0	52·4	<0·01
Triethylene melamine	Intrap.	0·16	1·6	104·0	<0·01
	Oral	0·20	2·0	2·0	0·20
	,,	0·40	4·0	1·4	0·20
	,,	4·00	4·0	3·6	<0·02>0·01

(b) *Fasting test animals.*—As shown in Table II, a single oral dose of T.E.M. or HN3 was almost inactive, but the same dose given to tumour-bearers which had been kept fasting for 22 or 32 hours before and 4 hours after treatment was significantly active. This dose, though tolerated by non-fasting rats, was toxic to the fasting animals (Table III).

TABLE III.—*Influence of Fasting on the Oral Activity of Single Doses of Triethylene Melamine and Nitrogen Mustard (Young Rats of 50 ± 3·7 g. Body-weight).*

Agent.	Dose (mg./kg. B.W.).	Test animal.	No. of survivors.	Change in body-weight (%).	Effect.	
					Ratio.	Signif. (P.).
Triethylene melamine	4·0	Fed	12/12	+52	3·6	<0·02>0·01
	4·0	Fasting	8/12	+40	27·0	<0·01
HN3 hydrochloride	2·0	Fed	12/12	+31	2·4	<0·02>0·01
	2·0	Fasting	12/14	+33	8·6	<0·01

Influence of the age of the test animals.

All the tests described above were carried out on young rats. Some further tests were performed with the adult group to find whether the permeability of the gastro-intestinal mucosa decreases with maturation. Some of the results varied with the age-group. After oral treatment with HN3 the weight ratios between control and treated tumours were greater in the young rats. In adult rats 2 mg. per kg. retarded tumour-growth slightly but significantly; 2·8 and 3·2 mg. per kg. produced an effect similar to that produced in the young animals by 2 and 2·8 mg. per kg. respectively. It is doubtful whether these differences were due to a greater permeability of the gastro-intestinal mucosa in the young rats, for after oral treatment with HN2 the ratios were greater in the adult rats, but not in the young rats (Table IV). Moreover, a difference in response between the two age

TABLE IV.—*Tumour-inhibitory Activity of Nitrogen Mustards and Triethylene Melamine Administered to Young and Adult Rats.*

Agent.	Route.	Daily dose (mg./kg. B.W.).	Total dose (mg./kg. B.W.).	Effect young rats.		Effect adult rats.	
				Ratio.	Signif. (P.).	Ratio.	Signif. (P.).
HN2 hydrochloride	Oral	0·28	2·8	5·4	<0·02>0·01.	15·6	<0·01
	,,	0·40	2·8	12·1	<0·01	25·3	<0·01
	,,	0·40	2·8			15·6	<0·01
HN3 hydrochloride	Intrap.	0·10	1·0	7·8	<0·01	1·7	
	,,	0·10	1·0			8·6	<0·01
	Oral	0·20	2·0	6·5	<0·01	2·9	<0·02>0·01
	,,	0·20	2·0	6·6	<0·01	3·5	<0·01
	,,	2·00	2·0	2·3	0·02	3·2	<0·01
	,,	2·00	2·0	2·4	<0·02		
	,,	0·25	2·5			4·4	<0·01
	,,	0·40	2·8	13·9	<0·01	7·2	<0·01
	,,	0·40	2·8	14·3	<0·01		
	,,	0·40	3·2			14·0	<0·01
Triethylene melamine	Intrap.	0·16	1·6	104·0	<0·01	346·0	<0·01
	Oral	0·40	4·0	1·4	0·2	1·6	0·1

groups was also found after intraperitoneal treatment: T.E.M., injected in a total dose of 1·6 mg. per kg., suppressed tumour-growth even more strikingly in

the adult group ; the ratio was about 3 times that recorded in the young animals. Seven tests only were performed in duplicate, six of them against the Walker tumour. In the three tests with the young rats the duplicate results agreed, whilst in the three tests carried out with the adult rats the duplicates agreed in two but disagreed in the other (intraperitoneal treatment with HN3). In view of the smaller number of tests carried out in duplicate one cannot exclude the possibility that the difference in response between the two age-groups may represent only variations in susceptibility to the tumour-inhibitors between the individual batches of animals.

Bromine substitutes.

In the case of haloarylalkylamines with little or no tumour-inhibitory activity, it was found that the replacement of chlorine by the more reactive bromine produced active compounds (Haddow, Kon and Ross, 1948). Bromine substitution in HN2 and HN3, however, did not increase the activity against the Walker tumour ; in fact, given in the doses in which the chlorine compounds were active the two bromine compounds showed no inhibitory activity whatever.

Toxicity of the nitrogen mustards and triethylene melamine in tumour-bearers.

Given orally, R 48 was tolerated in a dose about 36 and 50 times the maximum tolerated doses of HN2 and HN3 respectively. The maximum tolerated oral dose of *nor* HN2 was about 10 times larger than that of HN2 and 14 times larger than that of HN3 (Table II). The therapeutic ratio of HN2 and HN3, whether the drugs were given intraperitoneally or orally, seemed as low as 2, or even lower. As estimated from the death-rate, HN2 and HN3 were more toxic to tumour-bearing than to normal rats. HN2, for example, in a daily intraperitoneal dose of 0·28 mg. per kg. was tolerated by normal rats for 18 days but proved toxic to tumour-bearerrs within 10 days. Oral treatment with HN3 in a daily dose of 0·28 mg. per kg. was tolerated by normal rats for 14 days, whilst 0·25 mg. per kg. was toxic to tumour-bearers after 10 days. Toxicity was somewhat unpredictable in tumour-bearers : in one test, for instance, injections of HN2 in daily doses of 0·27 mg. per kg. were tolerated, whilst in two others 0·2 mg. per kg. was toxic. HN3 given orally in a total dose of 3·2 mg. per kg. was tolerated by one batch of tumour-bearers, though individuals from other batches were killed by 2·5 mg. per kg.

Goldin, Goldberg, Ortega and Schoenbach (1949) demonstrated a similar variation in mice bearing Sarcoma 180 ; in four batches injected with HN2 (7·5 mg. per kg. mouse) mortality varied from zero to 25 per cent. The young rats were at least as resistant to toxicity as were the adults, and in some instances they seemed even more resistant. For instance, oral treatment with HN2 in 7 daily doses of 0·4 mg. per kg. was toxic to adult but tolerated by young tumour-bearers.

Effect of nitrogen mustards and triethylene melamine on body-weight.

The tumour-inhibitory effect of the agents investigated was frequently associated with inhibition of somatic growth. In some instances, more frequent in the adult than in the young rats, there was even loss of mean body-weight. Animals treated orally with R 48, T.E.M. or *nor* HN2 gained in body weight considerably more than those treated with HN2 or HN3. The stunting effect of the

drugs appeared to be influenced by the distribution of the dose. Tumour-bearers which had received *nor* HN2 in a single oral dose (28 mg./kg.) gained in mean body-weight only 22 per cent less than the controls, but when the total dose was divided up over 10 daily doses, the growth rate of the treated rats was only about one-quarter of that of the controls. Similar differences were observed after oral treatment with HN2 or HN3. The maximum tolerated dose prevented body growth when divided into 10 daily doses, but allowed some gain in mean body-weight (28 to 52 per cent) when given in a single dose. Comparison of the effects of maximum tolerated doses given in the same distribution suggests that oral treatment suppresses somatic growth less than do injections.

Influence of adrenocortical extract or urethan on toxicity of HN3.

The addition of adrenocortical extract (Kucharik and Telbisz, 1945) or urethane (Anslow, Karnofsky, Jager and Smith, 1947) has been reported to reduce the toxicity of mustards. An attempt at reducing the toxicity of HN3 in this way was unsuccessful. The experiment is described here because it has been suggested that urethane potentiates the effect of nitrogen mustards clinically. Three groups of adult bearers of Walker tumour received HN3 orally in a daily dose of 0·4 mg. per kg. rat for 8 days. One group received in addition intraperitoneal injections of 25 mg. of urethane daily, a dose of urethane which *per se* is known to be inactive against the Walker tumour. Another group received unstandardized adrenocortical extract (Eucortone) intraperitoneally, 1 ml. (corresponding to 75 mg. of fresh gland) being given daily at the same time as the HN3. The toxicity of HN3 was not reduced by these collateral treatments; some animals died, and the survivors lost more weight than those which had received HN3 alone. The weight ratio between control tumours and those treated with HN3 + urethane (22) was greater than that between controls and those treated with HN3 alone (14), but the difference was statistically not quite significant ($P<0{\cdot}05>0{\cdot}02$) and does not prove a potentiating effect.

DISCUSSION.

The inhibition of growth of the Walker tumour produced by oral adminstration of aliphatic nitrogen mustards was comparable to that produced by administration of these reagents parenterally. *Nor* HN2 appeared to be at least as active as and at the same time less toxic than HN2 or HN3. Oral treatment with triethylene melamine, on the other hand, produced no significant inhibition of tumour-growth unless given to fasting rats, but to these a dose which was tolerated by fed rats proved toxic. This result is not at variance with those of previous workers. Hendry, Homer, Rose and Walpole (1951) found triethylene melamine orally active against the Walker tumour, but the figures published by these authors show that the oral doses were 10 to 20 times greater than those which they had found markedly active after injection. Paterson and Boland (1951) found the haematological response to oral doses less predictable than to parenteral treatment. Clinical trials by Karnofsky, Burchenal, Armistead, Southam, Benstein, Craver and Rhoads (1951) showed that the drug reacts readily with organic material in the stomach and is therefore almost inactivated unless given during fast.

As described above, treatment began one day after implantation of the test tumours. Nevertheless, the growth rate of the treated transplants differed little

from that of the controls during the first few days. Inhibition of the Walker tumour became manifest during the second week, suggesting that the agents did not influence the " take " of the transplants.

The therapeutic ratios of HN2 and HN3 are probably not larger after oral than after intraperitoneal administration.

In non-tumour-bearing rats fed with HN3, Roberts (1952, personal communication) found changes in the intestine very similar to those described by several authors after injections of nitrogen mustards (Selye, 1950). In view of this finding, it is worth noting that most of the tumour-inhibition shown in this paper was associated with significant suppression of somatic growth, whether the drugs were given intraperitoneally or orally. That held true even when the net weight (body-weight minus tumour-weight) was considered instead of the gross weight. Thus the question arises to what extent the tumour-inhibition recorded after oral administration of the agents was due to a specific effect or to starvation. Walpole (1951) has found 40 per cent growth-inhibition of untreated Walker tumours when somatic growth was prevented by underfeeding. If this 40 per cent of the observed tumour-inhibition is deducted from the figures in Tables I to IV, the maximum tolerated oral dose of HN3 would appear to be inactive in adult tumour-bearers (Table IV) whilst the significance of the other results remains unchanged. It may be assumed, therefore, that the retardation of tumour-growth described above was mainly due to other causes than starvation. This view is supported by the finding that the suppression of tumour-growth after a single oral dose of R 48 or *nor* HN2 was not associated with suppression of somatic growth. Moreover, whilst effects on tumour-growth were similar, effects on body-growth were more pronounced after intraperitoneal than after oral treatment. On the other hand, Elson's (1951) recent work suggests that specific tumour-inhibition may be " one manifestation of a more general growth-inhibiting action". In 14-day tests, tumour-inhibitors, including radiation, proved more active against the Walker tumour on a 5 per cent than on a 20 per cent protein diet. Most of the animals on both diets lost weight during the treatment, and the ratio between the weight losses in rats with the 20 per cent and the 5 per cent protein diet was almost the same as the ratio of the degrees of tumour-inhibition. According to Houck, Crawford, Bannon and Smith (1947), animals treated with nitrogen mustards lose body weight by loss of protein and water. The rats under these experiments, therefore, though fed with a high protein diet (19·2 per cent), lost protein. Loss of protein may also intensify the effect of inhibitors on tumour- and body-growth. According to Elson (1951), as an initially large dose of an inhibitor is gradually reduced, the animal will recover from the inhibitory action quicker than the tumour will, and the latter may completely regress under high protein diet. It may well be that in those of my experiments in which the drug was given in a single dose the loss of protein ceased in time to allow the animal to recover its growth-rate whilst tumour-growth remained retarded.

SUMMARY.

Administered intraperitoneally in about maximum tolerated doses, *nor* HN2, HN2 and HN3 had approximately equal activity against the Walker carcinosarcoma, although against the Sarcoma 37, HN3 was more active than HN2. The Walker tumour was significantly more affected by triethylene melamine than by the aliphatic nitrogen mustards. When tested against the mouse sarcoma,

however, triethylene melamine was more active than HN2, but not more active than HN3.

R 48, *nor* HN2, HN2 and HN3 given orally in about maximum tolerated doses had an inhibitory action against the Walker tumour similar to that following intraperitoneal injection. R 48 was at least 4 times as active as the aliphatic amines. Bis(2 chloroethyl)p-phenylenediamine was active after intraperitoneal but inactive after oral administration.

Bromine substitution in HN2 and HN3 seemed to cause loss of activity.

Triethylene melamine given orally was practically inactive in the non-fasting rat, whereas the same dose given to the fasting animal had marked activity but was toxic.

Tumour-bearing rats tolerated R 48 and *nor* HN2 in doses respectively 36 and 10 times the maximum tolerated dose of HN2. The toxicities of HN2 and HN3 were somewhat unpredictable and greater than in normal rats, whether the drugs were injected or given orally. An attempt at reducing the toxicity by the addition of adrenocortical extract or urethan proved unsuccessful.

I am indebted to E. Boyland, W. A. Broom, J. Craigie, Sir J. Drummond, W. E. Gye, A. Haddow, P. C. Koller and C. Chester Stock for their help and advice during the course of this work.

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THE CARCINOGENIC AZO- COMPOUNDS: CHEMICAL CONSTITUTION AND CARCINOGENIC ACTIVITY.

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Received for publication May 23, 1952.

SOON after the discovery of the carcinogenic activity of 1:2:5:6-dibenzanthracene by Cook, Kennaway and colleagues, Yoshida (1932, 1933, 1934) and Sasaki and Yoshida (1935) reported that the addition of *o*-aminoazotoluene to the food leads to the production of malignant liver tumours in rats. During the past two decades scores of other azo- compounds have been tested for carcinogenic activity of this type and many have been shown to be active. For reviews see Shear (1937), Kinosita (1937), Cook (1939, 1943, 1948), Cook and Kennaway (1938).

These carcinogenic azo- compounds form a very interesting group. Unlike the polycyclic aromatic hydrocarbons, they do not, as a rule, produce tumours at the site of injection, but mostly affect the liver. Furthermore, the induction of liver tumours with azo- compounds is markedly influenced by the diet, and if a " protective " diet is supplied, the production of tumours can be strongly inhibited or even entirely prevented.

All the azo- compounds are, of course, artificial synthetic substances; and they are highly coloured (mainly orange to red). Some are used as textile dyestuffs, and certain members of the group are used for colouring foodstuffs. For the most part such compounds are probably harmless (Cook, 1948). Most of the food-colouring matters which have been tested have failed to induce liver tumours in rats; and in any case it is most unlikely that anyone could ever consume enough of an azo- compound in food to have any deleterious effect. Nevertheless the risk remains, a risk which has recently been underlined by the report that benzeneazo-β-naphthol is a hepatotropic carcinogen when injected subcutaneously in mice (Kirby and Peacock, 1949).

It has also been suggested that azo- compounds may be responsible for some of the occupational cancers which are found among workers in the dyestuffs industry, and this is a problem which still requires further investigation.

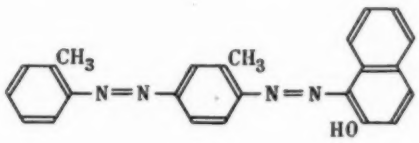
The study of the azo- compounds is of importance in any research into the process and mechanism of carcinogenesis. This review is an attempt to assess the present position in the study of the relationship between chemical constitution and carcinogenic activity in this series.

Historical.

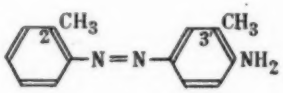
In 1906 Fischer described the atypical epithelial proliferation which resulted following the injection of a solution of scarlet red (I; also known as Biebrich scarlet R medicinal) into the ears of rabbits. The growths always receded and

never became malignant ; nevertheless, this was the first recorded instance of the production of a tumour-like proliferation by a pure chemical compound of any type. At Fischer's suggestion, scarlet red soon came into use to accelerate wound healing.

Not long afterwards it was found that the active part of the molecule is *o*-aminoazotoluene (II), also known as 4′-amino-2:3′-azotoluene, as 2′:3-dimethyl-4-aminoazobenzene (IIa), and as 2-amino-5-azotoluene (IIb). This compound was found to have an effect on epithelial cells similar to that of scarlet red, and it was likewise effective in accelerating healing (Hayward, 1909 ; Stöber, 1909).



(I)



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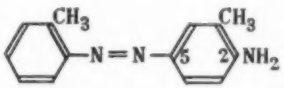


(IIb)

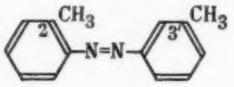
Malignant liver tumours in rats were first produced with this compound by Yoshida (1932, 1933, 1934) and Sasaki and Yoshida (1935), who administered it by addition to the food. Soon afterwards Shear (1937) found that subcutaneous implantation of the pure solid in pure strain mice also gave transplantable liver-cell carcinomas ; but no tumours were produced at the site of injection.

The carcinogenic activity of *o*-aminoazotoluene to the livers of mice and rats has been confirmed many times under a variety of experimental conditions (Hartwell, 1941). To some extent the compound is also carcinogenic towards the bladder (Yoshida, 1935), and it is interesting that the deaminated compound, namely 2:3′-azotoluene (III), was found to be non-carcinogenic to the liver of the rat, but produced numerous papillomas in the bladder (Otsuka and Nagao, 1936).

Many derivatives of azobenzene, containing substituent amino- and methyl-groups, were tested for carcinogenic activity by Kinosita (1937). None of these produced cancer of the liver except *o*-aminoazotoluene, its mono-acetyl- and diacetyl- derivatives, and an isomer of *o*-aminoazotoluene, namely 4-dimethyl-aminoazobenzene (IV). The latter compound, which is also known as *p*-dimethylaminoazobenzene, and as *N*, *N*-dimethyl-*p*-aminoazobenzene, was formerly used as a food-colouring matter under the name of "butter yellow". It was found to be even more active towards rat-liver than *o*-aminoazotoluene, producing a higher percentage of tumours in a shorter time ; but it has since been shown that the relative activities are reversed in mice (Law, 1941 ; Kirby, 1945). 4-Dimethyl-aminoazobenzene has been more extensively studied than any other azo- compound and its carcinogenic activity has been widely confirmed (Hartwell, 1941).

The parent compound, 4-aminoazobenzene (V), has usually failed to produce liver tumours (Miller and Miller, 1948) ; but Kirby (1944, 1947 ; Kirby and

Peacock, 1947) has obtained tumours of the liver in rats in experiments of very long duration. Liver tumours have not, however, been produced in mice with this compound (Sasaki and Yoshida, 1935 ; Kirby, 1945).



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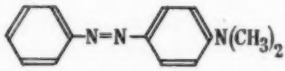

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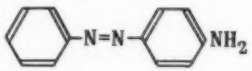
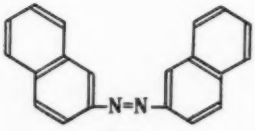


V

In recent years carcinogenic activity has been demonstrated in various derivatives of 4-dimethylaminoazobenzene, containing methyl-, fluoro-, chloro-, nitro- and other substituent groups. The effects of such structural alterations are described in a following section.

Most of the known carcinogenic azo- compounds are derivatives of 4-dimethylaminoazobenzene, or at least of 4-aminoazobenzene. Nevertheless, a 4-amino-group is not essential for activity of this type. This was shown for example, by Cook, Hewett, Kennaway and Kennaway (1940), who examined the action of azonaphthalenes on mice. The compounds were administered by subcutaneous injection, by painting on the skin, or by addition to the food. Many liver tumours, mostly of the type of cholangioma, were obtained with 2:2′-azonaphthalene (VI), and a few tumours were obtained with 1:1′-azonaphthalene (VII). No carcinogenic activity was, however, shown by 1:2′-azonaphthalene (VIII) or by its 4-amino- derivative.



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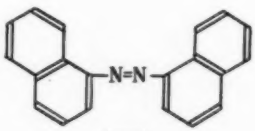

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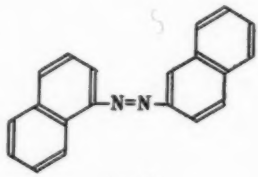


(VIII)

Relative Potency of Carcinogenic Azo- Compounds.

The carcinogenic azo- compounds vary considerably in potency as measured by the number of animals contracting the disease and the time taken to induce the tumours. However, it is difficult to assess the relative potencies with any degree of accuracy. Such difficulties are inherent in all biological assays, and are severely aggravated when one has to consider results obtained in several different laboratories, under different experimental conditions.

Rats have been most generally used in this work ; but mice have also been employed extensively, and tests have sometimes been carried out with other laboratory animals. Sometimes the azo- compound has been injected subcutaneously ; in other cases it has been administered in solution *per os* ; in very many experiments it has been administered by mixing with the food. As a matter of fact the latter method has been very widely used ; but it can hardly be considered very satisfactory for the evaluation of relative potencies. The exact dose is, of course, unknown, and comparatively slight alterations in

structure can have a marked effect on the palatability of the food-mixture, leading to marked variations in the total food intake, and hence the amount of azo-dye consumed.

So far, the most ambitious attempt to assess the relative potencies of carcinogenic azo- compounds has been that of Miller and Miller (1948), who have carried out a number of careful experiments in which the various compounds were added to the food in quantities equivalent (on a molecular weight basis) to 0·06 per cent of 4-dimethylaminoazobenzene.

Under the conditions used, 4-dimethylaminoazobenzene gave a tumour incidence of 70–92 per cent in rats after 4 months, and 100 per cent after 6 months. This compound was assigned an arbitrary relative potency of " 6," and the relative activities of other compounds assessed by the formula :

$$\text{Relative activity} = \frac{6 \times 4 \times \text{per cent tumours with test compound}}{\text{Months fed} \times \text{per cent tumours with 4-dimethylamino-azobenzene.}}$$

Compounds having relative potencies from *ca.* 1 to *ca.* 12 have been reported.

All compounds have not been examined under these conditions, however, and it would be quite impossible to assign a relative activity value of this type to every compound in the literature ; something simpler is clearly required.

In these circumstances 4-dimethylaminoazobenzene has been taken as the standard compound having " moderate " carcinogenic activity in the rat, and has been given the grading ++. Compounds having approximately equivalent carcinogenic activity have been given the same grading. Compounds having carcinogenic activity markedly greater than that of 4-dimethylaminoazobenzene in the rat have been graded +++ ; and similarly, compounds having less activity have been graded +. The symbol ± has been used for compounds exhibiting only trace activity, or activity which can only be demonstrated under very special dietary conditions. The symbol o has been used for those compounds which have not produced cancers of the liver ; but it must be remembered that many compounds given this grade are capable of producing other liver changes of a less serious nature.

The system adopted can best be appreciated by a study of Table I, which includes the relative potency values of Miller and Miller (1948).

TABLE I.—*Relative Potencies of Carcinogenic Azo- Compounds to the Livers of Rats.*

	Per cent tumour incidence at—					Relative potency.	
	3 months.	4 months.	6 months.	8 months.	10+ months.	Miller and Miller.	This paper.
3′-Methyl-4-dimethylamino-azobenzene	30	88a	..	..	..	10–12a	+++
4-Dimethylaminoazobenzene	0	70–92a	100a	..	..	6a	++
2′-Methyl-4-dimethylamino-azobenzene	..	..	..	44b	..	2–3c	+
o-Aminoazotoluene	..	..	..	..	30d	0c	+
4-Aminoazobenzene	0	0	0	0a	44e	0a	±

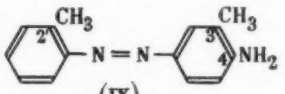
a, Miller and Miller, 1948. b, Miller and Baumann, 1945*b*. c, Miller, Sapp and Miller, 1949. d, Crabtree, 1949. e, Kirby, 1944, 1947.

The same relative potencies do not always apply in other laboratory animals. It is well known, for example, that 4-dimethylaminoazobenzene is much less active in producing liver tumours in mice than it is in rats. On the other hand, *o*-aminoazotoluene is much more effective in mice than in rats. The latter compound is therefore taken as the standard for the assessment of relative potencies in *mice*, and is assigned a grade of ++ for this species. In all other respects the system adopted is similar to that for rats.

The Carcinogenic Azo- Compounds.

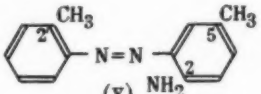
(i) *The aminoazo-toluenes.*

Malignant liver tumours in rats were first produced with 4-amino-2′:3-dimethylazobenzene or *o*-aminoazotoluene (IX), and this compound has always retained a prominent place in experimental carcinogenesis. In mice it is even more effective, and it seems to be the most potent of all the azo- compounds for this species. In addition, Law (1941) has reported that it produces sarcomas at the site of injection in susceptible strains of mice.

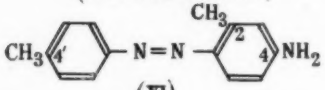


(IX)

(*o*-aminoazotoluene)



(X)



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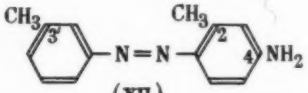

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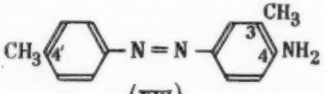

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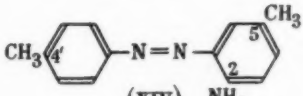
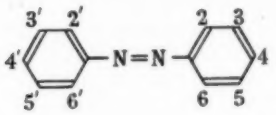


(XIV)

The structural isomers of this compound are of considerable interest. Six isomers have been examined by Crabtree (1949) under comparable conditions. Only two, 4-amino-2′:3-dimethylazobenzene (IX, 4′-amino-2:3′-azotoluene or *o*-aminoazotoluene) and 2-amino-2′:5-dimethylazobenzene (X, 2′-amino-2:5′-azotoluene) induced liver tumours in rats. In mice, however, malignant hepatomas were produced by 4-amino-2′:3-dimethylazobenzene (IX), by 2-amino-2′:5-dimethylazobenzene (X) and also by 4-amino-2:4′-dimethylazobenzene (XI, 4′-amino-2′:4-azotoluene). Slight activity was shown by the 4-amino-2:3′-dimethyl- and 4-amino-3:4′-dimethyl- azobenzenes (XII and XIII); but 2-amino-4′:5-dimethylazobenzene (XIV) proved to be innocuous (Table II).

The positions of the substituent groups are therefore of considerable importance; but it is clear that an amino- group in the 4-position is not essential for activity of this type.

TABLE II.—*Aminoazo-toluenes.*



Compound.	Carcinogenic activity.			
	Rats.	References.	Mice.	References.
4-Amino-2′:3-dimethylazobenzene	+	a	++	a
2-Amino-2′:5-dimethylazobenzene	+	a	++	ab
4-Amino-2:4′-dimethylazobenzene	o	a	++	a
4-Amino-2:3′-dimethylazobenzene	o	a	±	a
4-Amino-3:4′-dimethylazobenzene	o	a	±	a
2-Amino-4′:5-dimethylazobenzene	o	a	o	a

a, Crabtree, 1949. b, Kirby, 1945.

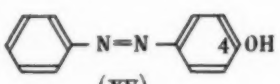
(ii) *Substituted azobenzenes.*

Azobenzene itself seems to be entirely inactive as a carcinogen ; but activity of various kinds has been demonstrated for many derivatives (Table III).

4-Hydroxyazobenzene (XV) produced papillomas in the stomach in rats, and 4-hydroxy-2′:3-dimethylazobenzene (XVI) gave papillomas of the bladder. The latter compound has also produced a number of sarcomas at the site of injection in susceptible strains of mice ; moreover it seems to have slight activity on the liver of this species (Law, 1941 ; Kirby, 1945). On the other hand, 4-chloro-2′:3-dimethylazobenzene seems to be inactive, at least in mice. The parent dimethyl-compound, 2′:3-dimethylazobenzene (XVII, commonly known as 2:3′-azotoluene), produced bladder papillomas when fed to rats in a rice diet (Otsuka and Nagao, 1936) ; but it does seem to be inactive towards the liver (Kirby, 1945). Law has also reported that it produces sarcomas at the site of injection in susceptible strains of mice.

Kinosita's discovery (1937) that 4-dimethylaminoazobenzene (XVIII) is even more potent in producing liver tumours in rats than *o*-aminoazotoluene has tended to concentrate attention on the 4-amino- and 4-alkylamino- derivatives of azobenzene, so that very little is known about the effect of other substituents.

As already mentioned, *o*-aminoazotoluene is a liver carcinogen both for rats and mice, and its monoacetyl- and diacetyl- derivatives have also produced liver tumours in mice.



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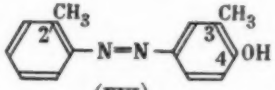
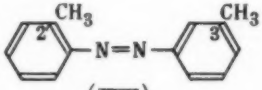


(XVI)



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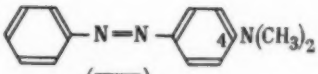


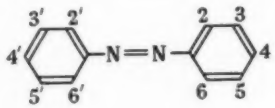
(XVIII)

4-Aminoazobenzene seems to have trace activity in the rat, and derivatives having methyl- substituents in the other ring are also either ineffective or only

slightly active. 4-Methylamino- and 4-dimethylamino-azobenzenes are, however, moderately potent carcinogens to rat liver. As a matter of fact it is not surprising that these compounds are approximately equally effective (Giese, Miller and Baumann, 1945), because Miller, Miller and Baumann (1945) have shown that 4-dimethylaminoazobenzene is partially demethylated *in vivo*, and also that 4-methylaminoazobenzene is partially *methylated*. (4-Aminoazobenzene, however, was not methylated.)

The higher *N*, *N*-dialkyl derivatives all seem to be ineffective as carcinogens (Sugiura, Halter, Kensler and Rhoads, 1945), although 4-diethylaminoazobenzene has also been shown to be dealkylated to 4-aminoazobenzene in rats (Kensler, Magill, Sugiura and Rhoads, 1946).

TABLE III.—*Substituted Azobenzenes.*



Compound.	Carcinogenic activity.			
	Rats.	References.	Mice.	References.
Azobenzene	o	a	o	a
4-Hydroxy-	o[1]	a	..	..
*4-Amino-	±	bc	o	i
4-Methylamino-	++	cde	..	..
†4-Dimethylamino-	++	abcde	+	ghi
4-Methylethylamino-	++	fj	..	..
4-Diethylamino-	o	d	o	b
4-Di-*n*-propylamino-	o	d	..	..
4-Di-*n*-butylamino-	o	d	..	..
4-Di-*n*-amylamino-	o	d	..	..
4-Phenylazo-	..	..	o	a
2:4-Diamino-	o	a	..	..
2:4-Diamono-5-methyl-	o	a	..	..
2:4-Diamino-2′:5-dimethyl-	o	a	..	..
4′-Methyl-4-amino-	+	d	..	..
2′-Methyl-4-methylamino-	+	f	..	..
3′-Methyl-4-methylamino-	+++	fj	..	..
4′-Methyl-4-methylamino-	+	f	..	..
2:2′:4′:5-Tetramethyl-4-amino-	o	a	..	..
2′:3′:4′:5′:6′-Pentamethyl-4-amino-	o	d	..	..
§2′:3-Dimethyl-	o[2]	a	±	ah
4-Chloro-2′:3-dimethyl-	..	..	o	a
4-Hydroxy-2′:3-dimethyl-	o[2]	a	±	h
4-Acetoxy-2′:3-dimethyl-	o	a	..	..
¶4-Amino-2′:3-dimethyl-	+	ak	++	ahklm
4-Acetylamino-2′:3-dimethyl-	+	a	..	..
4-Diacetylamino-2′:3-dimethyl-	+	a	..	..

*, i.e., *p*-aminoazobenzene. †, i.e., *p*-dimethylaminoazobenzene. §, i.e., 2:3′-dimethylazobenzene or 2:3′-azotoluene. ¶, i.e., *o*-aminoazotoluene.

[1], Papillomas of the stomach. [2], Papillomas of the bladdder.

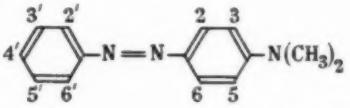
a, Hartwell, 1941. b, Kirby, 1944; 1947; Kirby and Peacock, 1947. c, Miller and Baumann, 1945*b*. d, Sugiura, Halter, Kensler and Rhoads, 1945. e, Giese, Miller and Baumann, 1945. f, Sugiura, 1948. g, Kinosita, 1937, h, Law, 1941. i, Kirby, 1945. j, Miller and Miller, 1948. k, Crabtree, 1949. l, Shear, 1937. m, Andervont, 1947.

The effect of substituents on the 4-dimethylaminoazobenzene molecule is considered in the next section ; but reference may be made at this stage to the effect of substituents on 4-methylaminoazobenzene. The 2′- and 4′-methyl-derivatives are *less* effective than the parent amine ; but the 3′- derivative is a potent carcinogen (Sugiura, 1945).

(iii) *Substituted* 4-*dimethylaminoazobenzenes.*

The pronounced activity of 4-dimethylaminoazobenzenes on the livers of rats has led to the preparation and testing of numerous derivatives of this compound (Table IV). These derivatives fall into two main classes.

TABLE IV.—*Substituted* 4-*dimethylaminoazobenzenes.*



Compound.	Carcinogenic activity. Rats.	References.
4-Dimethylaminoazobenzene	++	ab
2-Methyl-	o	bd
3-Methyl-	o	ab
2-Hydroxy-	o	b
2-Fluoro-	+++	e
2′-Hydroxy-	o	b
2′-Methyl-	+	bd
2′-Nitro-	+	ab
2′-Cloro-	+	ab
2′-Fluoro-	++	b
2′-Trifluoromethyl-	o	b
*2′-Carboxy-	+	c
3′-Hydroxy-	o	b
3′-Methyl-	+++	abd
3′-Nitro-	++	ab
3′-Chloro-	++	ab
3′-Fluror-	+++	b
3′-Trifluoromethyl-	o	b
3′-Ethoxy-	±	a
4′-Hydroxy-	o	b
4′-Methyl-	±	bd
4′-Nitro-	o	b
4′-Chloro-	+	ab
4′-Fluoro-	+++	b
4′-Trifluoromethyl-	o	b
4′-Arsonic acid	o	c
†4′-Sulphonic acid, sodium salt	o	c
2′:4′-Difluoro-	+++	e
2′:4′:6′-Trifluoro-	+++	e
2′:4′:6′-Trichloro-	o	b
2′:4′:6′-Tribromo-	o	b
3′:5′-Dimethyl-	o	a
2′:5-Dimethyl-	o	a
2′:4′-Dimethyl-	o	a

* Methyl red. †, Methyl orange.

a, Miller and Miller, 1948. b, Miller, Sapp and Miller, 1949. c, Hartwell, 1941. d, Miller and Baumann, 1945*b*. e, Miller, Miller and Sapp, 1951.

In the first, the additional substituent is on the same ring as the dimethylamino-group. Only a few such compounds have been tested, but, with the exception of the 2-fluoro- compound, all have been found to be inactive.

In the second class the additional substituent is attached to the other benzene ring. With the exception of the 2′-hydroxy- and 2′-trifluoromethyl- derivatives which are entirely inactive, the *ortho*- or 2′- substituted 4-dimethylaminoazobenzenes are all less active than the parent compound. Only the 2′-fluoro-derivative has about the same activity.

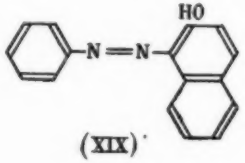
Among the *meta*- or 3′- substituted compounds, the hydroxy- and trifluoromethyl- derivatives are again inactive ; the ethoxy- derivative has trace activity ; the nitro- and chloro- derivatives have approximately the same activity as the parent amine ; and the 3′-methyl- and 3′-fluoro- derivatives are considerably more effective in producing liver tumours than the unsubstituted 4-dimethylaminoazobenzene.

The *para*- or 4′- substituted derivatives are mostly inactive, or only very slightly active ; but the 4′-fluoro- derivative is more effective than 4-dimethylaminoazobenzene itself. The 4′-methyl- and 4′-chloro- compounds are only slightly active, and the 4′-hydroxy-, 4′-nitro-, 4′-trifluoromethyl- and 4′-sulphonic acid derivatives are all completely inactive.

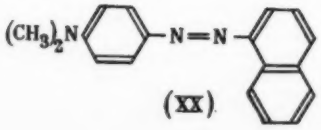
Relatively few di- and tri- substituted derivatives have been tested. It is of some interest, however, that the 3′:5′-dimethyl- derivative seems to be inactive (in spite of the fact that *mono*-methyl substitution at these positions *increases* the potency). Other dimethyl- derivatives are also inactive ; so are the 2′:4′:6-trichloro- and 2′:4′:6′-tribomo- compounds.

(iv) *Phenylazonaphthalenes and azonaphthalenes.*

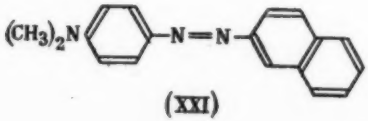
Many commercial dyestuffs and food-colouring matters fall into this group. Many of these are sulphonic acid derivatives, which are probably excreted too rapidly to be effective as carcinogens ; but some are simple hydroxy- or amino-derivatives. Among these, special interest attaches to 1-benzeneazo-2-naphthol (XIX), which is a food-colouring matter and which Kirby and Peacock (1949) have shown to be a liver carcinogen in mice. Very closely related to this substance are Sudan II, Sudan III, and scarlet red, substances which were used in early experiments and shown to promote the healing of wound tissue.



(XIX)



(XX)



(XXI)

It is also noteworthy that the 1- and 2-naphthalene analogues of 4-dimethylaminoazobenzene, namely 4-dimethylaminophenylazo-1′-naphthalene (XX) and 4-dimethylaminophenylazo-2′-naphthalene (XXI), are inactive in rats (Kinosita, 1940; Miller and Baumann, 1945*b*).

It is, however, the unsubstituted azonaphthalenes which form the most interesting series in this group. These compounds were originally examined because operatives engaged in the manufacture of naphthylamine are particularly liable to acquire cancer of the bladder. It was thought that azonaphthalenes might arise by mild oxidation of the naphthylamines, and might conceivably be present as impurities in the commercial product.

2:2′-Azonaphthalene (VI) was found to produce liver tumours in a very large proportion of the mice to which it was administered either by mouth, by subcutaneous injection, or even by painting on the skin. The tumours were mostly cholangioma, but some were hepatoma. A few tumours were also obtained with 1:1′-azonaphthalene (VII); but 1:2′-azonaphthalene (VIII) was found to be inactive (Cook, Hewett, Kennaway and Kennaway, 1940).

TABLE V.—*Phenylazonaphthalenes and Azonaphthalenes.*

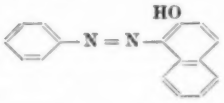
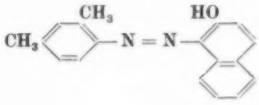
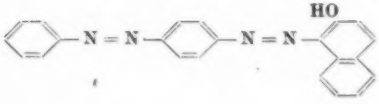
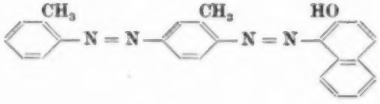
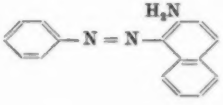
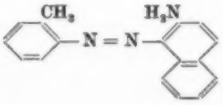
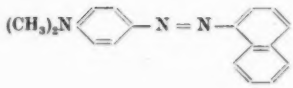
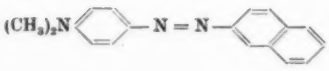
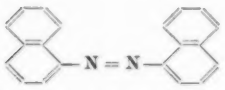
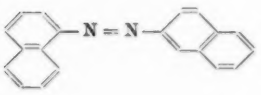
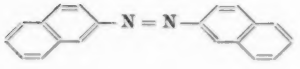
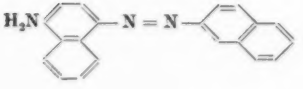
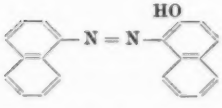
Compound.	Carcinogenic activity.			
	Rats.	References.	Mice.	References.
Sudan I ("oil orange E"), 	o	a	+	b
Sudan II, 	o	a		
Sudan III, 	o	a		
Scarlet red, 	o	a		
Yellow AB, 	..	..	o	c
Yellow OB, 	..	..	o	c

TABLE V.—(*cont.*)

Compound.		Carcinogenic activity.			
		Rats.	References.	Mice.	References.
	. . . . .	o	de		
	. . . . .	o	e		
	$\alpha\alpha'$	..	..	±	f
	$\alpha\beta'$	..	..	o	
	$\beta\beta'$	..	..	+++	
	$(\alpha\beta')$	..	..	o	f
	. . . . .	o	g		

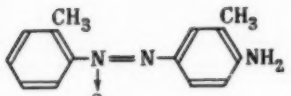
(Pigment Bordeaux N)

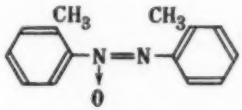
a, Hartwell, 1941. b, Kirby and Peacock, 1949. c, Badger, Cook, Hewett, Kennaway, Kennaway and Martin, 1942. d, Miller and Baumann, 1945*b*. e, Kinosita, 1940. f, Cook, Hewett, Kennaway and Kennaway, 1940. g, Maruya, 1938.

(v) *The influence of the azo-linkage.*

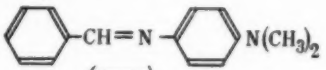
This can be studied in two ways : firstly by examining the effect of oxidation at the azo-linkage ; and secondly by progressive replacement of the nitrogen atoms by CH groups.

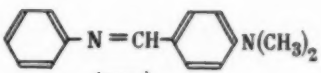
As to the first method, little has been done. It does appear, however, that 4-amino-2′:3-dimethylazoxybenzene (XXII), the azoxy- compound corresponding to *o*-aminoazotoluene, is inactive in rats (Nagao, 1939). Azoxybenzene and *o*-azoxytoluene (XXIII) are also inactive (Hartwell, 1941).

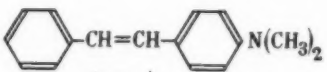

(XXII)


(XXIII)

The replacement of the nitrogen atoms by CH produces some curious results (Table VI). *N*, *N*-Dimethyl-*N'*-benzal-*p*-phenylenediamine (XXIV) and 4-dimethylaminobenzalaniline (XXV) are inactive in rats (Miller and Baumann, 1945b), but 4-dimethylaminostilbene (XXVI) has been shown to be a potent carcinogen. In mice, however, although tumours were produced, it proved to be much less potent. Several other compounds of the same type, including 2'-methyl-4-dimethylaminostilbene and α-(4-dimethylaminophenyl)-β-(1'-naphthyl)-ethylene are also potent carcinogens for the rat. Apart from sarcomas at the site of injection, these compounds produced many cancers at a distance, including carcinoma of the eyelid, cholangiomata of the liver, multiple mammary fibro-adenomata in females, adenomata of the lung, and so on (Haddow, Harris, Kon and Roe, 1948).


(XXIV)


(XXV)


(XXVI)

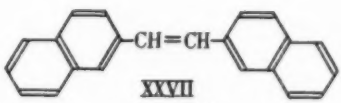
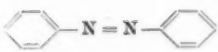
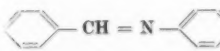
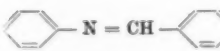
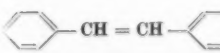

XXVII

TABLE VI.—*The Influence of the Azo-linkage.*

Compound.	Carcinogenic activity.			
	Rats.	References.	Mice.	References.
	++	a	+	c
	o	a		
	o	a		
	(++)	b	(+)	b

a, Miller and Baumann, 1945*b*. b, Haddow, Harris, Kon and Roe, 1948. c, Table III.

It seems therefore that the replacement of the azo-linkage in 4-dimethylamino-azobenzene produces a compound having less specialised carcinogenic activity.

On the other hand, it is noteworthy that 2:2′-dinaphthylethylene (XXVII) is completely inactive, in mice, in contrast to the striking activity of the corresponding azonaphthalene towards mice livers (Badger, Cook, Hewett, Kennaway, Kennaway and Martin, 1942).

The Effect of Diet.

It is not proposed to give a detailed review of the effect of diet on the carcinogenic activity of the azo- compounds; but a brief account is necessary (for a comprehensive review see Rusch, Baumann, Miller and Kline, 1945).

Nearly all the early work in this field was carried out in Japanese laboratories. The usual procedure was to add a solution of the azo- compound in olive oil to a measured quantity of ground rice. The rats were then fed this mixture, which was supplemented with a little carrot. Using this deficient diet, 4-dimethylaminoazobenzene was found to induce liver tumours in a high percentage of the rats used (Kinosita, 1937).

It soon transpired that the diet plays a very important role in this type of carcinogenesis. With more balanced diets the appearance of liver tumours was either delayed, or entirely prevented. The use of either wheat, rye or millet in place of rice afforded some protection; and various substances added to the basal diet were found to reduce the incidence of liver tumours to a marked degree. Beef liver and yeast were found to be especially effective in this respect (Nakahara, Mori and Fujiwara, 1939; Ando, 1938; Sugiura and Rhoads, 1941).

These observations indicated that vitamins of the B group might be involved, and tests have shown that while biotin, pyridoxine and vitamin B_{12} increase the incidence of liver tumours (du Vigneaud, Spangler, Burk, Kensler, Sugiura and Rhoads, 1942; Miner, Miller, Baumann and Rusch, 1943; Day, Payne and Dinning, 1950), riboflavin has a marked protective action. This protective action is greatest when the diet also contains adequate protein; nearly all the so-called protective diets are rich in both riboflavin and protein (Kensler, Sugiura, Young, Halter and Rhoads, 1941; Miner, Miller, Baumann and Rusch, 1943).

The protective action seems to be closely associated with the concentration of riboflavin in the liver. Rats fed diets known to accelerate tumour induction were found to have *less* riboflavin in the livers than controls; and rats fed various protective diets were found to have *greater* hepatic riboflavin levels than controls (Miller, Miller, Kline and Rusch, 1948). Moreover, certain azo- compounds markedly lower the hepatic concentration of riboflavin (Kensler, Sugiura and Rhoads, 1940). The decrease appears to be roughly equivalent to the carcinogenicity of the dye. Thus 3′-methyl-4-dimethylaminoazobenzene was most effective, 4-dimethylaminoazobenzene and 4-methylaminoazobenzene were fairly effective, and 2′-methyl-4-dimethylaminoazobenzene, 4′-methyl-4-dimethylaminoazobenzene, *o*-aminoazotoluene and 4-aminoazobenzene and azobenzene had little or no effect (Griffin and Baumann, 1946). Griffin and Baumann (1948) conclude that there is an inverse relationship between the rate of tumour development and the level of hepatic riboflavin maintained on any particular dietary regimen. Miller and Miller (1947) have found that when azo- compounds are fed to rats, a portion of the dye becomes very tightly bound in the liver. Especially significant was the observation that the amount of bound azo- compound found in the livers of rats fed 4-dimethylaminoazobenzene and 4-methylaminoazobenzene on

a low riboflavin diet was greater than that in rats fed on a high riboflavin diet. Furthermore, the potent carcinogenic compounds gave higher levels of bound azo-dye, in a shorter time than the less active compounds (Miller, Miller, Sapp and Weber, 1949).

In addition, Silverstone (1948) has shown that there is a direct relationship (Table VII) between the level of carcinogenic dye (in this case 4-dimethylamino-azobenzene) in the liver, and the incidence of liver tumours.

TABLE VII.—*Diet and Tumour Incidence.*

Diet.	Average level of carcinogenic azo-dye/g. of liver at 8 weeks' period.	Per cent incidence hepatomas at 6 months.
High protein, high fat . .	0·32 μg.	25
Brown rice, yeast . . .	0·22 „	37
Low protein, high fat . .	0·46 „	58
Low protein, low fat . .	0·51 „	71
Brown rice	0·89 „	96

It is reasonable to conclude therefore (i) that the greater the concentration the carcinogen can achieve in the liver, the greater its carcinogenic activity; and (ii) that a high riboflavin content in the liver reduces the concentration of carcinogen in that organ and hence reduces the tumour incidence.

The effect of the riboflavin may be to hinder the conversion of the azo-compound into its carcinogenic derivative (possibly a protein-azo- compound complex); or the riboflavin may increase the ability of the liver to detoxify the azo- compound by converting it into simpler non-carcinogenic compounds.

Metabolism of Azo- Compounds.

The carcinogenic azo- compounds do not, as a rule, produce tumours at the site of application in rats, but appear to have a more or less specific action on the liver. Some of the compounds do produce tumours in the urinary bladder and tumours at some other sites have also been observed. For example, Lowenhaupt (1949) observed some lymphoblastic lymphosarcomas in rats bearing intrasplenic pellets of 4-dimethylaminoazobenzene. Hoch-Ligeti (1949) obtained primary pancreatic tumours in rats fed 4-dimethylaminoazobenzene, and it seems that the dye can affect other organs if the liver is "protected" by a suitable diet. Nevertheless, the specificity is very striking and is in marked contrast to the carcinogenic polycyclic hydrocarbons such as benzpyrene and methylcholanthrene, which produce tumours at the site of application.

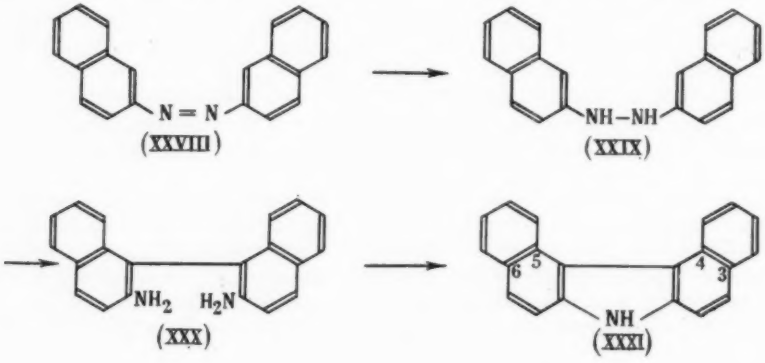
For this reason the metabolism of the azo- compounds is of considerable importance, and it has often been suggested that the azo- compounds are not carcinogenic *per se*, but that they are converted into active substances in the liver.

Most of the work in this field has been carried out with 4-dimethylaminoazo-benzene. This undergoes demethylation in the rat, relatively large amounts of 4-methylaminoazobenzene and 4-aminoazobenzene being recoverable (Kensler, Magill and Sugiura, 1947). Dealkylation of the diethyl- derivative also occurs (Kensler, Magill, Sugiura and Rhoads, 1946). It is also noteworthy that when 4-methylaminoazobenzene is administered, the liver has been found to contain

4-aminoazobenzene and 4-dimethylaminoazobenzene as well as the mono-methyl compound. On the other hand, when 4-aminoazobenzene was fed, this was the only dye found in the tissues (Miller, Miller and Baumann, 1945). Special methods for the determination of these compounds have been devised by Miller and Baumann (1945*a*).

4-Dimethylaminoazobenzene also undergoes reductive fission in rats, and *p*-phenylenediamine, *p*-aminophenol and their acetylation products have been detected in the urine (Stevenson, Dobriner and Rhoads, 1942). *p*-Phenylenediamine and *N*, *N*-dimethyl-*p*-phenylenediamine have been shown to inhibit certain important enzyme systems, and Kensler, Dexter and Rhoads (1942) therefore suggested that the carcinogenic activity may be due to " split products " of these types (or to a semi-quinone free radical of similar structure). In the opinion of the present reviewer this interesting hypothesis has now been proved erroneous. *p*-Phenylenediamine and its *N*, *N*-dimethyl- derivative have both been shown to be devoid of carcinogenic activity. Moreover, there are many azo- compounds which would be expected to yield these diamines on reductive fission, but which are entirely inactive or have only slight activity. For example, 4′-methyl-4-dimethylaminoazobenzene is much less active than 4-dimethylaminoazobenzene as a carcinogen, yet both would be expected to yield the same diamines. Similarly, it is surprising that 1′-(4′-dimethylaminophenylazo)naphthalene and 2′-(4-dimethylaminophenylazo)naphthalene are both entirely inactive (Table V). Many other examples could be given (Kirby, 1945; Crabtree, 1949; Rusch, Baumann, Miller and Kline, 1945). It is also significant that an amino- substituent is not essential for activity in an azo- compound, 2:2′-azonaphthalene being a striking example of an active carcinogen lacking such a group.

Another interesting hypothesis was put forward by Cook, Hewett, Kennaway and Kennaway (1940). It was suggested that the azo- compound may first be reduced to the hydrazo- derivative, and subsequently suffer the benzidine transformation and loss of ammonia to form a carbazole derivative. In particular, in the case of 2:2′-azonaphthalene (XXVIII) it was suggested that the true carcinogen, formed in the liver, is 3:4:5:6-dibenzcarbazole (XXXI). This transformation, illustrated by the scheme (XXVIII)⟶(XXXI), can certainly be carried out very readily *in vitro*, and although there is no proof that it does occur *in vivo*, certain facts are suggestive. It has been shown, for example, that the postulated intermediate diamine (XXX) produces liver tumours of the cholangiomatous type



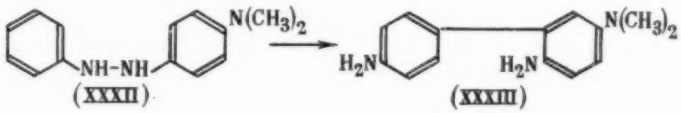
(XXVIII) (XXIX) (XXX) (XXXI)

(as does the azo-compound); and 3:4:5:6-dibenzcarbazole has not only been shown to produce liver tumours but also tumours at the site of application (Boyland and Brues, 1937).

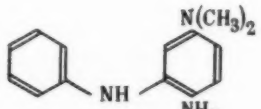
Evidence of a negative nature is provided by the related 1:2′-azonaphthalene and its transformation products. In this case the azo- compound, and the diamine derived from it, have little or no action on the liver. This diamine yields 1:2:5:6-dibenzcarbazole by prolonged boiling with hydrochloric acid, and this appears to be almost without carcinogenic action on the liver (Badger, Cook, Hewett, Kennaway, Kennaway and Martin, 1942).

Furthermore, Elson and Warren (1944) have found that when azobenzene is administered to rats, the urine contains not only aniline, but also a water-soluble compound (probably a derivative of hydrazobenzene) which on treatment with dilute acid is converted into benzidine. With 4-dimethylaminoazobenzene it seems that a benzidine type of rearrangement may take place *in vivo* even more readily than with azobenzene.

In spite of these successes, it is unlikely that this is the full story. Benzidine rearrangement of the hydrazo- compound (XXXII) resulting from 4-dimethylaminoazobenzene is known to give 2:4′-diamino-5-dimethylaminodiphenyl (XXXIII) together with some semidine (XXXIV) (Jacobsen, 1922); and the former has been shown to be inactive even when fed at a high level for 10 months. (In this connection, however, it is worth remembering that 4-dimethylaminodiphenyl produces cancer in a variety of organs in the rat (Miller, Miller, Sandin and Brown, 1949).



(XXXII) (XXXIII)



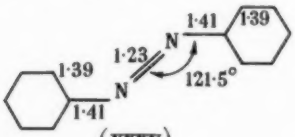
(XXXIV)

The theory is not obviously applicable to other substituted azo- compounds, especially as even closely related compounds often give different types of products in the benzidine reaction. 4-Aminoazobenzene, for example, unlike the 4-dimethylamino- derivative, gives the *p*-semidine as the major product (Jacobsen, 1922). Furthermore, Miller, Miller and Sapp (1951) have found that carcinogenic activity can be demonstrated in various substituted compounds which are unlikely to undergo the rearrangement. Only one of the five possible benzidine or semidine rearrangements could possibly occur in the case of 2′:4′:6′-trifluoro-4-dimethylaminoazobenzene (which is more potent than the parent amine); and this lone possibility involves a semidine rearrangement to the 2-position. The high activity of the 2-fluoro- derivative makes this possibility very unlikely. Miller, Miller and Sapp (1951) therefore maintain that although such rearrangements may occur in the metabolism of 4-dimethylaminoazobenzene, it is doubtful if they play any decisive role in carcinogenesis by this dye.

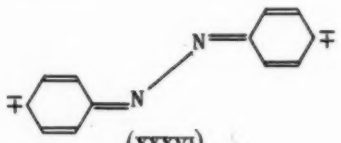
Chemical Constitution and Carcinogenic Activity.

Some azo- compounds are known to exist both in *cis* and *trans* forms ; but the *trans* form is always the more stable. All the compounds which have been tested for carcinogenic activity appear to have been *trans* compounds, so that this is the only configuration which need be considered here.

The structure of *trans* azobenzene itself has been established by analysis of the X-ray diffraction pattern (de Lange, Robertson and Woodward, 1939). It is a flat molecule, and has the dimensions shown in the accompanying diagram (XXXV). The C — N bonds (1·41 Å) are somewhat shorter than normal single bonds of this type (1·47 Å) ; and the N=N bond (1·23 Å) is somewhat longer than a normal double bond of this nature (1·20 Å). The explanation is, of course, that the azo- group is conjugated with the aromatic rings. In other words, structures of type (XXXVI) contribute to the " resonance hybrid". The C — N bonds therefore have some double bond character, and hence are shorter than pure single bonds. Similarly, the N=N bond has some single bond character, and hence is longer than a pure double bond of this nature. In related compounds e.g., azonaphthalenes) the character of the N=N bond is dependent on the extent of conjugation with the aromatic ring systems ; the greater the conjugation, the smaller the double bond character of the N=N bond.



(XXXV)



(XXXVI)

It must be remembered that each nitrogen atom has a " lone pair " of electrons not involved in bond formation. These electrons are also shared with the ring systems to some extent, so that azo- nitrogen atoms are relatively non-basic.

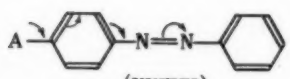
There is thus a certain electron density associated with the N=N linkage in azo- compounds, this density being controlled by the extent of conjugation with the particular aromatic ring systems involved.

Substituents can also affect this density of electrons, and it is instructive to consider this aspect of the matter in some detail.

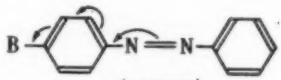
Substituents can be expected to modify the electron density at the N=N linkage in two ways. In the first place they may modify the extent of conjugation of the azo- group with the ring systems, and either increase or decrease the double bond character. It seems likely, however, that such modifications are of a minor character, and that the electron density will be affected to a greater degree by direct transfer of charge to (or from) one of the nitrogen atoms.

An electron-donating substituent (A) in the *ortho* or *para* position, for example, would increase the electron-density around the nitrogen further removed from it, as in structure (XXXVII). Similarly, an electron-attracting substituent (B) in the *ortho* or *para* position would decrease the density of electrons around the same nitrogen atom, as in structure (XXXVIII).

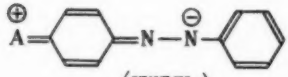
This is equivalent to saying that structures such as (XXXVIIa) and (XXXVIIIa) contribute to the resonance hybrids of such substituted compounds to a significant extent.



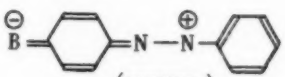
(XXXVII)



(XXXVIII)

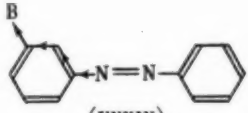


(XXXVIIa)



(XXXVIIIa)

Substituents in the *meta* positions, however, are not conjugated with the azo- group, and hence can affect the electron density only by virtue of their inductive effects. In this case, the *nearer* nitrogen atom will be more affected, as in structure (XXXIX).

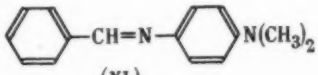


(XXXIX)

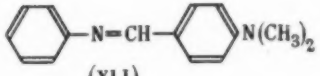
These effects of substituents are of importance because Pullman (1946 ; see also Pullman and Pullman, 1946) has suggested that carcinogenic activity in this type of compound is to be associated with a certain critical density of electrons on the azo-linkage (called the K′ position). This hypothesis is analogous to that for the polycyclic aromatic hydrocarbons, which associates carcinogenic activity with a critical electron density on the phenanthrene-type double bond, called the K position (Pullman and Pullman, 1946 ; Pullman, 1947 ; Badger, 1948).

Pullman (1947) divides the azo- compounds into three groups :

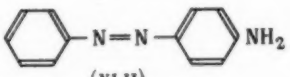
(*a*) Those compounds which are inactive or only slightly active by virtue of an insufficient electron density on the K′ region. This group includes compounds such as the anils (XL and XLI), 4-aminoazobenzene (XLII), and 4-diethylamino-azobenzene (XLIII).



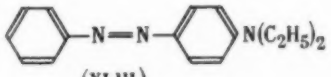
(XL)



(XLI)

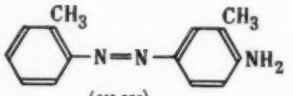


(XLII)

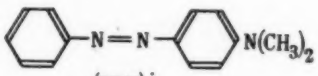


(XLIII)

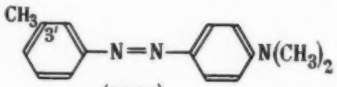
(*b*) This group includes the active compounds, that is, those substances having an electron density at the K′ position within the postulated upper and lower limits. Some examples are *o*-aminoazotoluene (XLIV), 4-dimethylamino-azobenzene (XLV), and 3′-methyl-4-dimethylaminoazobenzene (XLVI).



(XLIV)

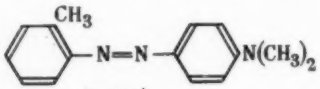


(XLV)

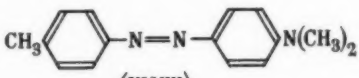


(XLVI)

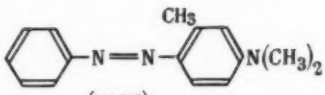
(*c*) The third group includes those which are only slightly active or inactive by virtue of their excessive electron density at the K′ position. Derivatives of 4-dimethylaminoazobenzene with a methyl group in one of the *ortho* or *para* positions (e.g., XLVII, XLVIII and XLIX) fall into this class.



(XLVII)



(XLVIII)



(XLIX)

Unfortunately the available data at present are too few for a really satisfactory examination of this interesting hypothesis. Very few azo- compounds having electron-donating substituents other than alkylamino- groups have yet been examined ; and the testing of a further series of unsubstituted compounds such as the phenylazonaphthalenes, azophenanthrenes, and so on, would also be of value.

The evaluation of the electron density at the K′ position by experimental methods is also necessary before any satisfactory comparison between electron density and carcinogenic activity can be made.

Hamon (1947) has studied the rates of hydrogenation of various azo-compounds ; but unfortunately this is not a very satisfactory method for the determination of electron densities at the K′ position. As she pointed out, every substituent in the azobenzene molecule, besides having an effect on the benzene ring, the other substituents, and on the azo-linkage, can also have an effect on the catalyst (in this case Raney nickel). In addition, there is the possibility that the reduction products of the different azo- compounds may poison the catalyst to varying degrees, and this retarding influence on the rate of reduction may vary from one azo- compound to another.

In a recent attempt to assess the influence of substituents, Rogers, Campbell and Maatman (1951) have determined the ionisation constants of some 4′-substituted 4-dimethylaminoazobenzenes. It seems that the first proton is added to an azo-nitrogen and the second to the dimethylamino-nitrogen. The first and second pKa values were determined, and the results indicate that the greater the electron-donating power of the 4′-substituent, the more easily is the proton added

(i.e., the stronger the basicity of the compound). The effect of the 4′-substituents on the proton affinity of the azo-nitrogen atoms was found to be in the same order as the net electron affinities of the groups as measured by Hammett's substituent constant, " σ " (Hammett, 1940).

This means that in a series of azobenzenes, the electron densities at the K′ position must be directly related to the substituent constants of the substituents. It should be possible, therefore, to examine the validity of the Pullman hypothesis by comparing the carcinogenic activities of, say, a series of 3′- and 4′- substituted 4-dimethylaminoazobenzenes with the substituent constants of the substituents. This is done in Table VIII, where the compounds are arranged in order of increasing basicity (increasing proton affinity). Too few compounds of this nature have been tested for any supposed correlation to be of significance ; but at least it may be said that the available results do support the suggestion that activity is associated with an optimum electron density (or proton affinity) at the K′ position. The 3′-ethoxy- and 4′-chloro- compounds are less active than might be predicted, but otherwise the correlation is as good as could be expected. (Of course, many other factors such as the rate of metabolic detoxication, etc. etc., may also be involved.)

It is not possible to extend this treatment to the 2′- or 2-substituted compounds as steric factors are of importance with such " *ortho* " derivatives, and no σ values can be determined. Similarly, substituents in the 3-position would be expected to interfere sterically with the dimethylamino- group, and such compounds cannot therefore provide any information of value for the present purpose.

TABLE VIII.—*Substituent Constants and Carcinogenic Activities of some 4′-substituted 4-Dimethylaminoazobenzenes.*

Substituent.	Substituent constant, σ.	Carcinogenic activity (rats).
4′-Nitro-	+·778	o
3′-Nitro-	+·710	++
3′-Chloro-	+·373	++
3′-Fluoro-	+·337	+++
4′-Chloro-	+·227	+
3′-Ethoxy-	+·15	±
4′-Fluoro-	+·062	+++
Parent compound	·000	++
3′-Methyl-	−·069	+++
3′:5′-Dimethyl-	−·138	o
4′-Methyl-	−·170	±

It should also be possible to examine the validity of the Pullman hypothesis by studying the rate of addition of electrophilic reagents to the K′ region in a series of carcinogenic and related non-carcinogenic azo- compounds. Other things being equal, the greater the electron density at the nitrogen atoms, the greater the reactivity towards electrophilic reagents. The peracids are known to be electrophilic reagents (Swern, 1947), and perbenzoic acid has therefore been used against a series of substituted azobenzenes, and azonaphthalenes Badger and Lewis, 1951 ; Badger and Lewis, 1952, unpublished results). The reaction was shown to be bimolecular and may therefore be represented by the equation :

$$R{-}N{=}N{-}R + Ph.CO_3H \longrightarrow R{-}\underset{\displaystyle O}{N}{=}N{-}R + Ph.CO_2H$$

Azobenzene, for example, gave azoxybenzene, and azonaphthalenes gave azoxynaphthalenes.

Among the non-substituted compounds, the different reaction rates must depend on the fact that the azo- group is not always conjugated with an aromatic ring to the same extent. As expected, and in agreement with the Pullman hypothesis, electron-releasing subtituents increase the reaction rate, and electron-attracting substituents retard the reaction (Table IX). For example, 4-methoxyazobenzene reacts much more rapidly with perbenzoic acid than azobenzene, and 4-carbethoxyazobenzene reacts more slowly than the unsubstituted compound. Halogenated azobenzenes also react more slowly than azobenzene itself, the inhibitory effect of a halogen in the 4- position being Cl>Br>F.

TABLE IX.—*Rates of Reaction of Perbenzoic Acid with Azobenzene and Related Compounds, at 25°.*

Compound.	Reaction rate $10^3k_2^{25°}$.
Azobenzene	13·9
4-Fluoroazobenzene	9·7
4-Chloroazobenzene	7·5
4-Bromoazobenzene	9·2
4-Methoxyazobenzene	58
1-Phenylazonaphthalene	14·5
2-Phenylazonaphthalene	14·5
1:1′-Azonaphthalene	2·5
1:2′-Azonaphthalene	17·7
2:2′-Azonaphthalene	17·6

In conclusion, although it seems impossible at present to come to any very definite decisions, recent work has been encouraging, and there is little doubt that future research will yield significant results on the relationship between chemical constitution and carcinogenic activity in this class of compound.

SUMMARY.

1. Many derivatives of azobenzene produce cancer of the liver when administered to rats and mice on a restricted diet.

2. The diet must be low in riboflavin and in protein.

3. Most of the active compounds are amino-, methylamino- or dimethylamino-derivatives of azobenzenes; but an amino- group is not essential for activity of this nature (e.g., 2:2′-azonaphthalene).

4. Additional substituents have variable effects depending on their nature and position.

5. The hypothesis that carcinogenic activity in this series is associated with an optimum density of electrons at the —N=N— linkage is supported by recent experimental work; but further research is necessary before any definite conclusions can be drawn.

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THE VALUE OF THE RABBIT FOR CARCINOGENICITY TESTS ON PETROLEUM FRACTIONS.

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Received for publication May 28, 1952.

THE experiments recorded in this article were carried out in the course of investigations on behalf of the Medical Research Council, a sub-committee having been set up in 1949 to inquire into the carcinogenic properties of mineral oils and allied products. The arrangements for co-ordinating the research have been briefly described by Auld (1950).

The entire series of tests have been made in duplicate at the Cancer Research Laboratories, Department of Pathology, University of Birmingham, and at the Chester Beatty Research Institute, The Royal Cancer Hospital, London. At each centre the activity of 3 selected crude oils, 4 fractions derived from each crude by methods of distillation specially designed to avoid high temperature cracking, and the final residues, have been tested on groups of 50 mice for each sample and also on a total of 105 rabbits. The results appear to be of particular interest, since it must be concluded from them that it is unsatisfactory to exclude carcinogenicity on the basis of tests on mice only.

The desirability of utilising in industry those types of oils which are least likely to cause dermatitis or cancer of the skin of workers has been recognised for over 20 years, and in 1934 the Committee on Cancer of Manchester Corporation proposed a standard for assessing the potency of mineral oils depending on physical properties (specific gravity and refractive index) which it was believed would ensure that the lubricants used in the textile industry would be substantially free from carcinogenic properties. This Manchester formula, which is not now regarded as a satisfactory criterion, was put forward following the investigations of Twort and his co-workers (Twort and Fulton, 1929 ; Twort and Twort, 1930, 1931, 1933 ; Twort and Lyth, 1939 ; Twort, 1941), who tested a wide range of crude mineral oils, distillates, solvent extracts, spindle and shale oils, using mice almost exclusively, however, for the biological assessment of their carcinogenicity. In the past, also, mice have for the most part been employed in skin tests with " oil " fractions derived from coal tars and with the pure chemical carcinogens either isolated or synthesised.

It is true that the classical experiments of Yamagiwa and Itchikawa (1918), in which the carcinogenicity of coal tar was first proved, utilised rabbits, and this species has also been employed in a number of early experiments on tar cancer, e.g., Bonne (1927), Leroux (1927), Babès (1929) and Twort and Twort (1930). Also a few early workers have used other species to a limited extent, e.g., rats and guinea-pigs (Watson, 1932). The majority, however, have favoured the mouse for large scale experiments, no doubt partly because of low maintenance costs and

availability, but probably influenced also by the experience of early workers that the skin of rats did not readily respond to coal tars, and that papillomata on rabbits so produced remained small and often regressed when applications ceased.

Thus the mouse has become accepted as the most sensitive test animal for such work ; the possibility that other species might prove more suitable under certain circumstances does not appear to have been sufficiently appreciated. Schurch (1939), however, found that his experiments with rabbits indicated that carcinogenic ingredients in addition to the recognised carcinogen 3 : 4-benzpyrene were probably present in certain coal tars. This was pointed out by Berenblum (1947), who demonstrated that the relative carcinogenic activity of a number of fractions, obtained by chromatographic procedures, varied greatly according to whether standardised against the skin of the mouse or of the rabbit. He had already shown (Berenblum, 1945*a*) that tumours were readily elicited on the rabbit by 9 : 10-dimethyl-1 : 2-benzanthracene, which was found to be more potent to rabbits than to rats or guinea-pigs, in which positive results were, however, obtained (Berenblum, 1945*b*). He also subsequently observed (1947) that this hydrocarbon when injected subcutaneously did not induce rabbit tumours, though rats and guinea-pigs responded by this method. Much earlier, Oberling, Sannié, Guerin and Guerin (1937) had shown that a 1 per cent solution of 3 : 4-benzpyrene in benzene was active on rabbits' skins, and Berenblum (1945*a*) observed that 9 : 10-dimethyl-1 : 2-benzanthracene was much more active than benzpyrene at a similar (0·5 per cent) concentration.

Experiments using a high boiling fraction of mineral oil from an experimental catalytic cracking operation have recently been carried out by Smith, Sunderland and Sugiura (1951), testing the residue after removing the lower boiling naphtha and light gas oil cuts, upon mice, rats, guinea-pigs, rabbits and rhesus monkeys. The rats and guinea-pigs were found to be refractory to skin applications but papillomata were elicited in all of the 6 monkeys, 2 being proved cancerous by biopsy 4 years after the start of the experiment. Papillomata were produced on the inner surface of the ears of the 21 rabbits within 100 days. The number and size of such growths tended to increase during the 2 years in which painting was continued, and in 3 of the 6 surviving animals cancerous changes in the growths were observed. For tests on the type of material which they intended to study, namely, samples containing oils which had been subjected to a process of fluid catalysis up to 950° F. in the presence of alumina or silica, these workers concluded that white mice were the best animals.

Recently Cruikshank and Squire (1950) obtained numerous papillomata on the ear and body skin of albino rabbits after applications of a " cutting oil " obtained from a machine sump. Only 1 benign papilloma was produced by this oil in a group of 46 mice, of which 50 per cent survived 40 weeks' and 28 per cent 52 weeks' treatment, and they suggested that such materials should be tested against both species before being regarded as non-carcinogenic to man.

In the present investigation it was decided that it was of importance that the properties of the mineral oils in question should be tested on both rabbits and mice.

Crudes and oil fractions.

The 3 crudes were obtained from Kuwait, Lagunillas and Oklahoma respectively and had the following characteristics :

Kuwait (Kuwait Oil Co.): an extensively produced Middle East crude likely to be much used in the United Kingdom, of paraffinic-asphaltic base and containing lubricating oil fractions.

Lagunillas Venezuela (Shell Petroleum Co.): a typical naphthenic crude yielding well-known lubricating distillates.

Oklahoma City Mid-Continental (Socony Vacuum Oil Co.): yields Mid-Continental distillates, bright stock and residual lubricating oils.

The derived fractions were produced by a research group appointed by the Institute of Petroleum, which has been responsible for the choice of the crudes having regard to their type, distribution and industrial importance. The initial fractionation of the 3 crudes was carried out at delimited temperatures using vacuum and steam in an apparatus selected to preclude cracking. These operations were carried out at the Thornton Research centre, Shell Refining and Marketing Co. Ltd., for the Institute of Petroleum.

Full details regarding the physical characteristics of these samples are not pertinent to the purpose of this communication, but will be presented in due course in connection with the wider aspects of the investigations.

Experimental arrangement and procedure.

Mouse tests.

For each fraction 50 animals 10 to 12 weeks of age were housed in metal boxes $11 \times 8\frac{1}{2} \times 4\frac{1}{2}$ inches, 5 in each box. At Birmingham they were selected, 25 of each sex, from an outbred laboratory albino strain previously employed in grading a series of oil fractions (Woodhouse and Irwin, 1950). In London the mice used were from laboratory stocks randomised so that each group of 50 contained some of each colour and genetic constitution. They were fed on Thomson's cube diet with addition of oats and a little green food. Water from bottles was always available.

The oils were applied twice weekly for 52 weeks to the inter-scapular region using approximately 0·2 ml. on each occasion. All applications were made by the same person throughout for each centre. Records of deaths, appearance of papillomata, etc., were kept, and the skin from the treated area of all mice which survived 12 weeks' treatment were prepared for histological examination. Animals surviving were killed after 52 weeks.

Rabbit tests.

It was not possible to obtain the requisite number (105) of animals of one inbred strain, and at Birmingham medium-sized males of Dutch breed with erect ears and predominantly brown or grey in colour were randomised. In London the rabbits were males of mixed commercial stock, with agouti, Flemish giant, black and albino well represented. They were housed in fairly small cages of usual type and fed on greens alternated with crushed oats and a little bran. Applications of the oils to patches of skin about 3 cm. square from which the hair had been removed by electric clippers were made twice weekly. Six areas on each of 30 animals were used for testing the 3 crudes, and 75 animals were used for the 15 derived fractions employing 7 areas on each animal. The 6 areas comprised the 2 ears, left and right thoracic, and the left and right lateral abdominal. The seventh area was the inter-scapular region. These sites are shown in Fig. 1.

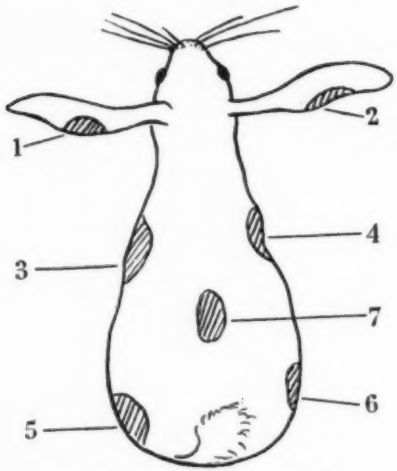

FIG. 1.—Painting sites on rabbits.
Owing to an error in the drawing of this figure, Area 7 has been placed too low and should be higher between the scapulae.

The scheme for allocating the fractions and crudes to the various areas was in accordance with the arrangement shown in Tables I and II, which were devised

TABLE I.—*Distribution of Crude Oils on Rabbit Sites* (*Fig.* 1).

Rabbits.	Sites.					
	1.	2.	3.	4.	5.	6.
1–5	L	L	K	K	O	O
6–10	K	K	O	O	L	L
11–15	O	O	L	L	K	K
16–20	L	L	O	O	K	K
21–25	K	K	L	L	O	O
26–30	O	O	K	K	L	L

L = Lagunillas crude, O = Oklahoma crude, K = Kuwait crude.

TABLE II.—*Distribution of Derived Fractions and Residues on Rabbit Skins* (*Fig.* 1).

Rabbits.	Sites.						
	1.	2.	3.	4.	5.	6.	7.
31–35	L3	K3	O4	LR	OR	L4	K4
36–40	O1	O3	L4	K2	O2	O4	OR
41–45	OR	K1	L2	KR	O4	L1	L4
46–50	O2	K2	K4	K1	L1	LR	O4
51–55	O4	LR	KR	O1	L2	K4	O3
56–60	K2	O4	L3	L2	KR	K3	O2
61–65	L1	O1	K3	O4	O3	L3	K1
66–70	K4	L2	O2	L1	L4	O3	L3
71–75	K1	L3	K2	L4	K4	O1	KR
76–80	KR	L4	O3	O2	K3	K1	LR
81–85	L4	L1	O1	K3	LR	K2	L2
86–90	L2	O2	K1	K4	O1	OR	K3
91–95	K3	K4	OR	03	K2	KR	L1
96–100	LR	KR	L1	OR	L3	O2	O1
101–105	O3	OR	LR	L3	K1	L2	K2

R = Residue.

by Dr. J. O. Irwin (Medical Research Council's Statistical Research Unit, University of London), so that a statistical evaluation of the effects of site variation and differences in response by the individual animals might be possible. It will be apparent that each crude was applied to 60 sites and each of the other fractions to 35 sites.

Applications were made twice weekly using approximately 0·3 ml. for each. It was necessary to remove hair approximately every 10 days to ensure clear areas. This was done with special care to avoid scratching the skin. Much less trouble than had been anticipated was encountered from the animals spreading the oil to other areas of the body, though some difficulty resulted because some patches became caked with thick layers of dandruff. Records were made of the condition of the sites throughout the 52 weeks, and finally the appropriate skin areas were preserved for histology.

RESULTS.

The general results for all the mice and rabbit series are set out in Table III. In addition to the mouse tumours recorded in this Table, with several fractions a number were observed which regressed after a short period and had not recurred up to the time of death or at the fifty-second week, in spite of continued applications

TABLE III.—*Results Obtained from the* Birmingham *Laboratories* (= *B*) *compared with those from* London (= *L*).

	Oil (Fractions arranged in order, light→heavy).		Rabbit test: Yield of tumours. B/L.	Mouse test: Yield of tumours. B/L.
Kuwait	Crude oil	K	0/0	2/0
	Light fraction	K1	1/0	2/0
	↓	K2	3/1	4/1
		K3	5/4	3/0
	Heavy fraction	K4	12/8	2/1
	Residue	K5	0/0	1/0
Lagunillas	Crude oil	L	0/0	0/0
	Light fraction	L1	1/0	2/0
	↓	L2	4/2	5/0
		L3	13/3	5/2
	Heavy fraction	L4	16/6	3/1
	Residue	L5	0/1	0/0
Oklahoma	Crude oil	O	0/2	0/0
	Light fraction	O1	0/1	0/0
	↓	O2	1/2	0/0
		O3	1/4	2/1
	Heavy fraction	O4	4/4	2/1
	Residue	O5	0/1	0/0

Although such spontaneous regressions are not uncommon, the frequency has been a particular feature of these tests at both centres.

It will be observed that the total number of mouse tumours in the Birmingham series was 33/900 and only 6/900 in the London series, although the survival rate was better in the latter. The greatest number observed with any fraction was 5. Thus the activity of all these materials was very slight in the mouse tests; the Birmingham results suggest that the Lagunillas derived fractions were somewhat more active than the Kuwait and that the Oklahoma set were essentially inert.

In contrast, there was a total of 61 tumour-bearing rabbit sites in the Birmingham series and 39 in the London series out of a possible 705 sites in each case, or, excluding the crudes and residues, which altogether yielded only 4 tumours (London series), the remaining 12 fractions produced 61 and 35 tumours in the Birmingham and London series respectively on a possible 420 sites. Thus the skin of the rabbit was distinctly more sensitive to these fractions than that of the mouse. This observation was confirmed by the type, rate of growth and other characteristics of the papillomata ; for example several tumours appeared on one rabbit site, and this is also corroborated by full statistical analyses of the results with reference to survival rate of the mice, the effect of site distribution in the rabbits, etc., which have been undertaken by Dr. Irwin.

DISCUSSION.

Both in the rabbit and in the mouse tests a somewhat higher incidence of tumours was obtained at Birmingham than at London. This may possibly be due to a difference in the strain of animals, for Smith and Sunderland (1951) have observed differences in the response of different strains of mice. It is impossible to exclude the possibility of slight differences in technique. Considering that the strains of mice were different in the two laboratories, the agreement between the Birmingham and the London results is better than might have been expected. Although the absolute yield of tumours is different in the two cases, the relative activity of the different fractions shows an appreciable consistency (Table III).

To account for the greater activity on rabbit skin the possibility that these mineral oil fractions may contain types of carcinogen differing chemically from the polycyclic hydrocarbons hitherto encountered must be entertained. In this connection it is important to emphasize that the cracked oils used by Smith, Sunderland and Sugiura (1951) would probably constitute a series containing considerable quantities of different types of hydrocarbons from those present in the fractions utilised in the present investigation. From the oils employed by the American investigators, compounds such as isopropyl-1 : 2-benzanthracene and methyl chrysene were isolated, while the presence of pyrene and benzphenanthrene derivatives was also demonstrated by Fischer, Priestley, Eby, Wanless and Rehner (1951).

The extraordinary complexity of mineral oils and the minute proportion of carcinogenic components in the uncracked oils must present very considerable difficulties in any effort to isolate or characterise them. Twort (1951) reported preliminary attempts to do this but they were not continued. Further experiments using both species of animals to test fractions selected from these crude oils are being carried out which it is believed will provide further information and facilitate future attempts to identify the carcinogenic agents.

SUMMARY.

Tests have been carried out in two laboratories to determine the carcinogenic action to the skin of mice and rabbits of three typical mineral oil crudes and 15 fractions derived from them by processes avoiding cracking. The activity on mice of all the fractions was very slight ; some, particularly those with a boiling range of 325 to 375° C., showed high activity on the rabbit ear and body skin.

Both series of experiments demonstrated, therefore, that the rabbit was more

sensitive to these types of oil than the mouse, and it is concluded that this species should be included in carcinogenic tests on this type of material.

The Chester Beatty Research Institute receives grants from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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THE DISTRIBUTION OF MITOSES IN A FIBROBLAST COLONY.

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Received for publication June 3, 1952.

FOR the development of quantitative methods of study of mitotic inhibition by X-rays and chemical agents, it has been necessary to examine the distribution of mitoses in tissue cultures of chick fibroblasts. All the resting and mitotic cells forming the outgrowth of a typical fibroblast colony have been counted and their topographical distribution recorded. A statistical analysis of the distribution of the mitoses has been made.

Previous quantitative work on tissue culture colonies *in vitro* has been reviewed by Mayer (1939). Mitoses in fibroblast colonies were mapped by Fischer and Parker (1929), by Gaillard (1935), by Jacoby (1937), and in epithelial colonies by Ephrussi and Litvac (1934). Of these only Gaillard's and Jacoby's maps are directly comparable with the present work. Willmer (1933) studied the relationship between cell density and frequency of mitosis. Since 1939 the results of extensive studies of fibroblast colonies grown in a two-phase medium have been published by Cunningham and Kirk (1942) and Tompkins, Cunningham and Kirk (1947), but these colonies are not directly comparable with "hanging drop" colonies.

MATERIAL AND METHODS.

The culture studied was kindly supplied by Mrs. I. Simon-Reuss. It was a hanging drop colony of the 4th passage from a culture of fibroblasts from the choroid and sclerotic of an 11-day chick embryo. The colony had been grown for 24 hours in the usual mixture of one drop of fowl plasma and one drop of 15 per cent chick embryo extract, then fixed in Susa, stained with Heidenhain's haematoxylin, and mounted whole. It was selected from a number of "control" colonies of another experiment, as being of average growth and mitotic activity, and free from any obvious abnormality.

The colony was mapped using the 1/6 inch (dry) objective and an eye-piece micrometer which divided the field of view into 16 squares each of 90μ side. The number of cells in each square was counted, and at the same time a record was made of cells in mitosis, and of the phase of mitosis. The region counted was the whole of that part of the colony in which the resting cells could be distinguished and counted, and included all the squares in which the number of cells was less than about 60, and a few where it was over 60. It probably corresponded to Mayer's (1939) Zones IV and III, and part of Zone II.

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RESULTS.

The result of the count is presented in the form of a contour map (Fig. 1), by which the colony is divided into five arbitrary zones according to the cell density. The area of the uncounted central part is 22 per cent of the total area of the colony. The distribution of resting cells does not show any remarkable features ;

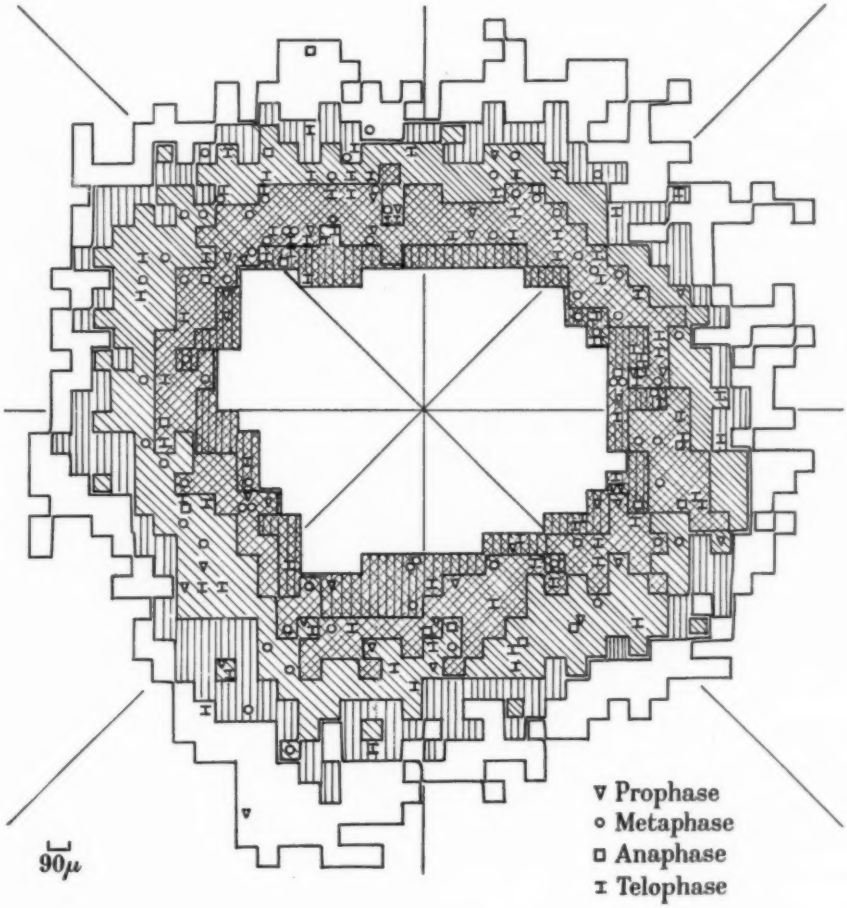

FIG. 1.—Map of Colony 3897. The mitoses are marked in their correct squares, but the position with the 90 μ square is arbitrary. 1–4 cells per 90 μ square ; 5–9 cells per 90 μ square ; 10–19 cells per 90 μ square ; 20–34 cells per 90 μ square ; 35 and over cells per 90 μ square.

the cell density falls off fairly steadily from the central part outwards. The distribution of mitoses is summarised in Table I, which shows that the number of

TABLE I.—*Summary of Distribution of Mitoses.*

Zone.	Cells per square.	Number of squares.	Number of cells.	Number of mitoses.	Mitoses per 1000 cells.	Mitoses per mm.^{2}.
1	1–4	229	459	4	8·7	2·2
2	5–9	134	951	10	10·5	9·2
3	10–19	206	2,842	66	23·2	39·5
4	20–34	164	4,360	87	20·0	65·5
5	35+	78	3,635	41	11·3	64·8
Whole area	—	811	12,247	208	17·0	31·7

mitoses per 1000 cells is highest in the region of moderate cell density and falls off both towards the centre and towards the periphery, while the number of mitoses per unit area rises from the periphery to reach a steady value in the two zones nearest the centre. The proportions of the phases of mitosis over the whole area are: prophase 17 per cent, metaphase 34 per cent, anaphase 7 per cent, telophase 42 per cent, and within the limits of statistical variation they are the same in each of the five zones.

In order to detect any departure from random distribution of mitoses within any one zone, the numbers of squares containing 0, 1, 2, 3, . . . etc. mitoses were compared with the numbers expected on the Poisson distribution, and were found to agree within the limits of statistical significance. There was a suggestion that the number of squares containing more than two mitoses was slightly greater than expected, but the total numbers were too small to establish that this was more than random variation. A similar comparison for the separate phases of mitosis is shown in Table II, and again there is no significant departure from the Poisson distribution. The agreement between observed and expected numbers suggests that chance is the most important factor in the distribution.

TABLE II.—*Distribution of Individual Phases Compared with Poisson Distribution (Zones 4 and 5 together).*

Number of mitoses in block of 4 squares.	Prophase.		Metaphase.		Anaphase.		Telophase.		Total.	
	Observed.	Expected.	Observed.	Expected.	Observed.	Expected.	Observed.	Expected.	Observed.	Expected.
0	38	35·7	22	25·4	44	43·1	18	21·8	7	5·7
1	10	14·2	23	18·7	7	8·9	25	19·4	13	12·7
2	4	2·8	8	6·9	2	0·9	8	8·6	15	14·2
3	1	0·4	0	1·7	0	0·06	2	2·5	7	10·5
4	0	0·04	0	0·3	0		0	0·6	6	5·85
5	0		0	0·05	0		0	0·1	2	2·6
6	0		0		0		0		1	1·0
7	0		0		0		0		2	0·3
8 and over	0		0		0		0		0	0·1

TABLE III.—*Comparison of Octants (Zones 4 and 5 together).*

Octant number.	Number of squares.	Number of mitoses		χ^2.
		Observed.	Expected.	
1	27	13·5	14.3	0·04
2	29	27·5	15·4	9·52
3	39·5	23	20·9	0·21
4	27·5	11	14·6	0·89
5	32	9	16·9	3·69
6	24	9	12·7	1·08
7	27·5	12·5	14·6	0·30
8	35·5	22·5	18·8	0·73
Total	242	128	128·2	16·46

$\chi^2 = 16{\cdot}46$; $n = 7$; $p = 0{\cdot}02$.

Finally, the inner two zones have been divided into octants, and the distribution of mitoses among the octants has been compared with the expected distribution on the basis of area (Table III). The total value of "chi squared" is 16·5, which, for 7 degrees of freedom, gives a probability $p = 0{\cdot}02$. This strongly suggests a factor other than chance causing non-uniform distribution. The greatest departure from uniformity is the relative excess of mitoses in the second octant. There is no departure from the general proportions of the phases, and therefore the cause is less likely to be synchronous cell division than a local factor favouring mitosis.

Comparison with Previous Work.

Gaillard's (1935) comprehensive study includes two points which can be compared with the present work, namely, the mitotic frequency per 1000 cells, and the number of mitoses per unit area. Mayer (1939) quotes from a personal communication from Gaillard, the mitotic frequency per 1000 cells in successive zones from the outermost inwards towards the central part (which was not counted), as 69·0, 52·5, 17·4, 7·6, 5·2 respectively. This is a very different trend from that found in the present work. The number of mitoses per unit area can be seen in the map of his colony 27646 to be lower in the zone nearest the central part than the average value by at least a factor of 2, which again is in marked contrast with the present work.

The map published by Jacoby (1937) can be compared as regards the relative number of mitoses per unit area in the different parts of the colony, and shows a similar trend to that found in the present work. It is interesting to note that the fairly constant level reached outside the central zone is maintained in Jacoby's colony across the central zone itself. If this was also the case in the present colony, there would have been about 120 mitoses in the central part which was not counted.

Willmer (1933) found that the frequency of mitosis was greatest in regions of moderate cell density, as is the case in the present colony.

SUMMARY.

The distribution of cells and of mitoses in the outgrowth of a typical fibroblast colony is presented in the form of a map. The cell density rises steadily from

the periphery of the colony inwards. The maximum mitotic index is in the region of moderate cell density, and the number of mitoses per unit area rises from the periphery to a steady value near the central part. The distribution of mitoses within the regions of constant average mitotic frequency agrees in general with a random distribution, which suggests that chance is the main factor controlling it. The relative proportions of the different phases of mitosis are the same in all parts of the colony. There is a significantly increased mitotic frequency in one sector. These findings are compared with other published work.

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EXPERIMENTS ON THE MECHANISM OF ACTION OF TETRA-SODIUM 2-METHYL-1:4-NAPHTHOHYDROQUINONE DIPHOSPHATE AS A MITOTIC INHIBITOR AND RADIOSENSITISER, USING THE TECHNIQUE OF TISSUE CULTURE. EXPERIMENTAL METHODS AND QUANTITATIVE RESULTS.

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Received for publication June 3, 1952.

AN attempt is being made to improve the results of the radiotherapy of some types of cancer by the ancillary use of chemical agents designed to modify the effects of radiation on the metabolism of cells. Laboratory studies and clinical trials of large doses of tetra-sodium 2-methyl-1:4-naphthohydroquinone diphosphate (Compound I) mainly as a radiosensitiser in conjunction with palliative X-ray therapy in various types of advanced malignant tumours have been in progress since 1946 (Mitchell and Simon-Reuss, 1947; Mitchell, 1948, 1949*a*, 1949*b*, 1950, 1951).

The selection of Compound I was based on experiments with chick fibroblast cultures, in which it was shown that Compound I produced mitotic inhibition in concentrations in the region of 4×10^{-6}M, and that potentiation of the effects of X-radiation and of Compound I in inhibiting mitosis appeared to occur under suitable conditions.

Tissue culture provides a very sensitive experimental method which can be applied quantitatively to study the direct effects produced by chemical agents and ionising radiations on cells growing *in vitro*.

This paper is an account of further work on the use of chick fibroblast cultures to examine the mechanism of action of Compound I, and deals mainly with the development of quantitative experimental methods and the analysis of the potentiation by Compound I of the mitotic inhibition and abnormal mitosis produced by X-rays.

METHODS.

(i) *Tissue culture technique.*

The tissues used were taken from the choroid and sclerotic of 10 to 11-day chick embryos. The cultures were grown by the hanging-drop technique in a medium consisting of equal parts of fowl plasma and 15 per cent chick embryo extract in Tyrode solution. The explants were subcultured every 48 hours for 4 passages to obtain as uniform growth as possible. Fresh embryo extract was used. Groups of cultures, each from the 4th passage, were selected and matched and used for experimental material and controls. The compounds to be tested were added usually in aqueous solution to the embryo extract to produce the required final molar concentration. The cultures were incubated at 39° C. for different times, usually 3, 6, 12 and 24 hours after the 4th subcultivation, and then

fixed in Susa solution. The plasma clot around the culture was pricked and rinsed several times with Susa solution before floating the coverslip on the fixative for 8½ minutes. In this way the stainability of the clot was reduced by removing much of the serum and leaving mainly the fibrin network supporting the cells. The cultures were stained with Heidenhain's iron haematoxylin.

Most of the information was obtained from counts of the total number of cells in mitosis in the outgrowth (zone of growth, Mayer, 1939, p. 67) in the control and treated cultures. In all cases the cells in mitosis were classified according to the phase as prophase, metaphase, anaphase or telophase.

Attention was paid to abnormal cells with special reference to cell enlargement, cytoplasmic changes including vacuolation, impaired division of the cytoplasm, chromosome fragmentation, chromosomes with regions of impaired stainability and irregular and beaded structure, anaphase bridges with and without visible fragments, clumping of chromosomes, irregularities of timing of the mitotic processes, spindle abnormalities, including irregular and defective formation of the spindle and multipolar mitoses, and cell degeneration and cytolysis. Photomicrographs were taken of many abnormal cells for detailed study.

Two methods have been used in the case of Compound I to study the possible role of cell migration. The areas of the outgrowth of the cultures were measured by planimetry of outlines drawn with a camera lucida, with linear magnification 27·5. Further, serial paraffin sections of thickness 10μ were made vertically through 24-hour cultures, and counts of resting and mitiotic cells made in the central parts of control cultures and of cultures treated with 5×10^{-6}M compound.

In some cases the effects of the chemical agent were observed on the unfixed living cells in culture by cine-photomicrography. For most of the work we used apparatus modified from that described by Willmer (1933). Recently, in collaboration with Dr. A. F. W. Hughes of the Department of Anatomy, we have recorded the effects on the living cells by phase-contrast ciné-photomicrography, using the techniques described by Hughes (1949, 1952).

Phase contrast microscopy has been used in a few cases to observe directly the effects of the chemicals on the living cells in culture.

(ii) *Radiological details.*

The primary X-radiation used was of equivalent wave-length 0·22Å, as deduced from the H.V.L. 0·40 mm. copper (Grebe and Nitzge, 1930; Lamerton, 1940; Greening, 1947). The apparatus was a 220 kV_p " Maximar " unit working at 15 ma. The additional filter employed was 1 mm. aluminium.

The doses given were measured with backscatter and represent nominal doses in soft tissue. The nominal dose rate, which was always measured within $\pm$ 2 per cent was in the region of 200r per minute at distance 40 cm. from the focus, and was varied by altering the distance. The quality of the radiation was unchanged throughout these experiments, so that the problem of the exact value of the ionisation in the cells of the culture near the surface of the glass coverslip is not of importance here. In the cultures used most of the mitotic cells counted lie between the glass surface and a distance 20μ from it, so that the ionisation in the cells is increased by photo-electrons arising in the glass, probably by a factor in the region of 2 (Spiers, 1949). A possible source of error is variation in the effective distance of the cells from the surface of the glass in different cultures.

(iii) *Preparation used.*

Tetra-sodium 2-methyl-1:4-naphthohydroquinone diphosphate, Compound I, of molecular weight 422.12, is a water-soluble synthetic vitamin K substitute.

The preparation used was "Synkavit" manufactured and kindly given by Roche Products Limited. As preservative, the preparation contains Nipagin (methyl ester of p-hydroxybenzoic acid) 0·08 per cent w/v, Nipasol (n-propyl ester of p-hydroxybenzoic acid) 0·01 per cent w/v, and potassium metabisulphite 0·1 per cent w/v. In a concentration corresponding to 1×10^{-5}M Compound I, the preservative was found to produce no mitotic inhibition or cytological abnormalities in the chick fibroblast cultures under the usual experimental conditions.

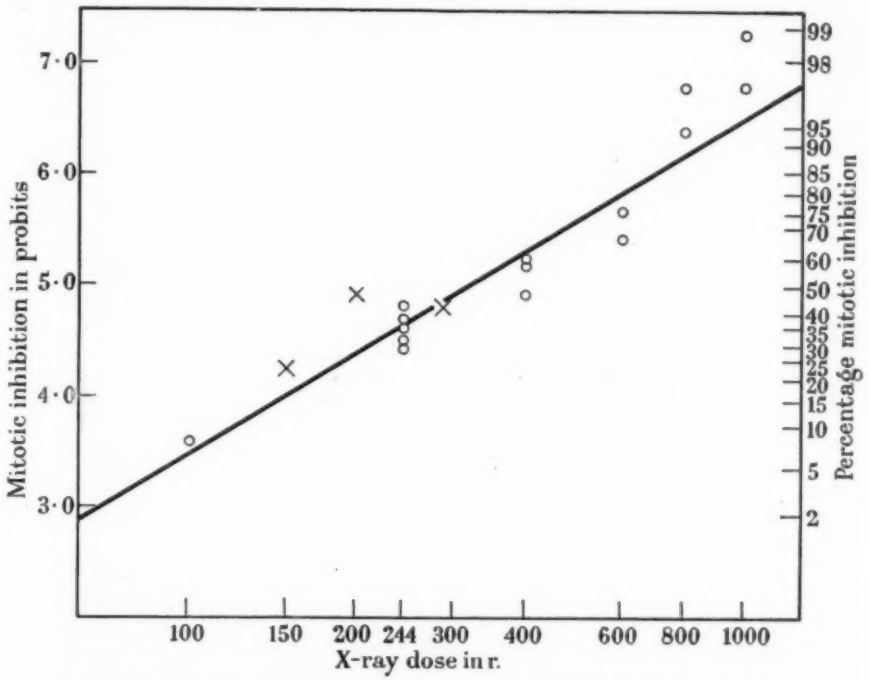


FIG. 1.—Mitotic inhibition by X-radiation in chick fibroblast cultures after 6 hours. The X-radiation was of equivalent wave-length 0·22Å. The doses were given in a fixed time 2·00 minutes except in the case of the three points marked ×, for which the radiation was given at 200 r per minute.

There is a linear relation between the mitotic inhibition in probits and log dose.

For 50 per cent mitotic inhibition, the dose is $315 \begin{smallmatrix} + 25 \\ - 22 \end{smallmatrix}$ r.

Potentiation by Compound I of Mitotic Inhibition Produced by X-Rays.

As the basis for the study of potentiation, it was necessary to measure the mitotic inhibition produced by X-radiation and by I acting separately on the chick fibroblast cultures under the present experimental conditions.

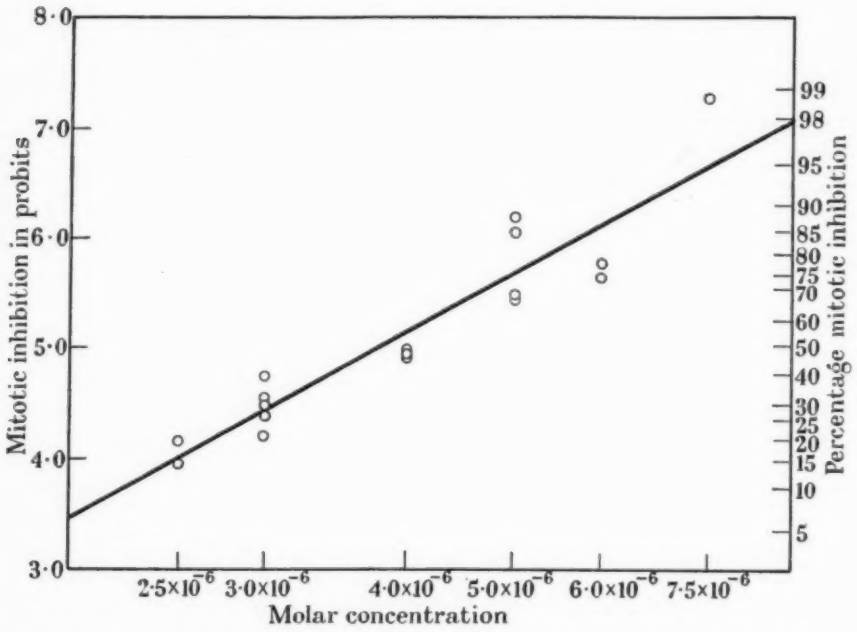


FIG. 2.—Mitotic inhibition by tetra-sodium 2-methyl-1:4-naphthohydroquinone diphosphate (Compound I) in chick fibroblast cultures after 24 hours.

There is a linear relation between the mitotic inhibition in probits and log concentration. For 50 per cent mitotic inhibition the concentration is $3{\cdot}81 \pm 0{\cdot}15 \times 10^{-6}$M.

EXPLANATION OF PLATES.

Fig. 3, 4, 5.—Effect of tetra-sodium 2-methyl-1:4-naphthohydroquinone diphosphate (Compound I) on a living resting cell of a chick fibroblast culture of the fourth passage, 24 hours old.

Phase-contrast ciné-photomicrographs, taken by Dr. A. F. W. Hughes. × 1500.

Fig. 3.—Immediately before addition of the compound.

FIG. 4.—A few seconds after addition of the compound in concentration 4×10^{-6}M. Slight transient shrinkage.

FIG. 5.—The same cell 46 minutes later. Cell enlargement, involving cytoplasm, mitochondria, nucleus and nucleoli.

FIG. 6, 7, 8, 9, 10.—Effects of tetra-sodium 2-methyl-1:4-naphthohydroquinone diphosphate (Compound I) in concentration 3×10^{-6}M on cells of chick fibroblast cultures. Susa fixation; Heidenhain's iron haematoxylin. × 850.

FIG. 6.—After 6 hours. Enlarged cell in prophase.

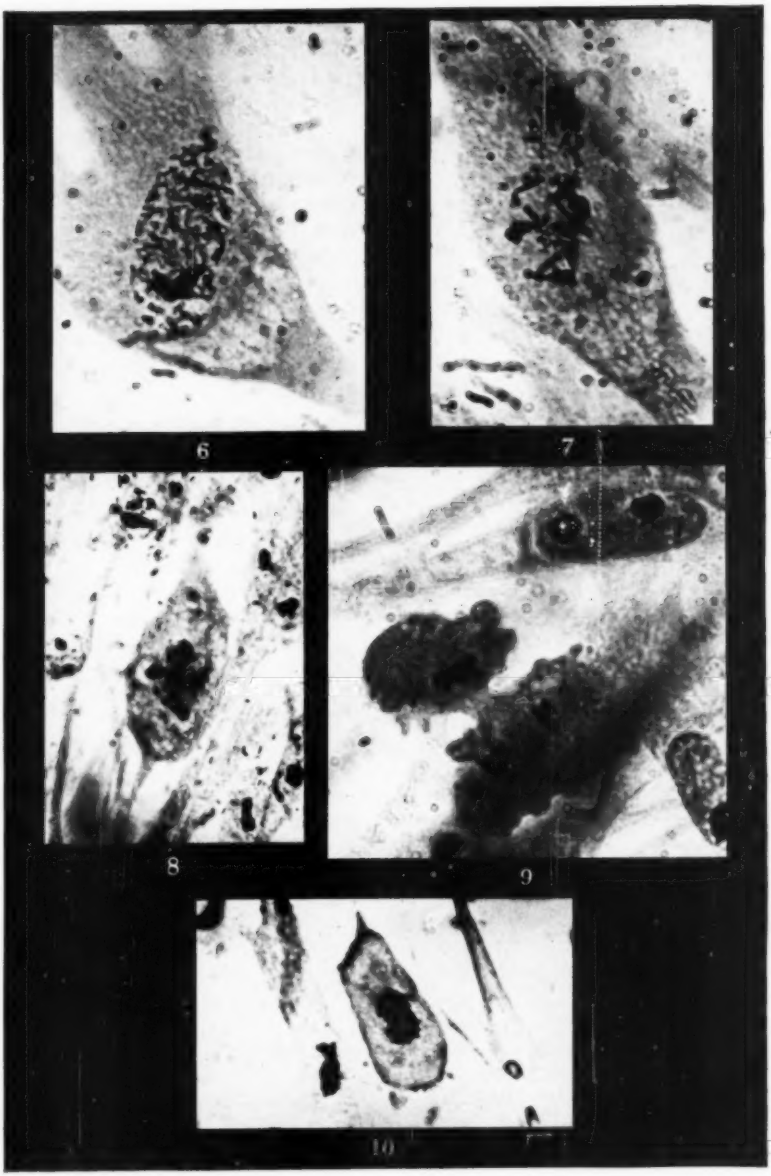
FIG. 7.—After 6 hours. Enlarged cell in prometaphase with chromosome fragmentation and chromosomes showing regions of impaired stainability and irregular and beaded structure.

FIG. 8.—After 24 hours. Metaphase with clumping and irregular distribution of chromosomes, and some cytoplasmic enlargement.

FIG. 9.—After 24 hours. Enlarged cells; a tripolar telophase and resting cells.

FIG. 10.—After 24 hours. Application of compound followed after 18 hours by 244 r. X-radiation delivered in 2·00 minutes. Typical anaphase bridges.

Mitchell and Simon-Reuss.



Mitchell and Simon-Reuss.

Many studies of the effects of X- and γ-rays on chick fibroblast cultures have been made (Spear, 1935; Lasnitzki and Lea, 1940; Gray, Mottram, Read and Spear, 1940; Paterson, 1942; Simon-Reuss and Spear, 1947). The information now required concerns the relation between the mitotic inhibition observed after 6 hours and the dose of X-radiation, together with evaluation of the dose corresponding to 50 per cent mitotic inhibition. The results of the present experiments are summarised in Fig. 1. Each of the 18 points is obtained from mitotic counts on 4 to 6 irradiated cultures (usually 6 except for breakages) and 6 control cultures. The doses were given in a fixed time, 2·00 minutes, except in the case of the three points marked, for which the radiation was delivered at 260 r per minute. The mitotic inhibition in probits is plotted against log dose (Finney, 1947; Fisher and Yates, 1948) The calculated regression line gives a satisfactory fit. There is no indication of departure from a linear relation between mitotic inhibition in probits and log dose. It is concluded that the relation between mitotic inhibition and the radiation dose plotted on a logarithmic scale is a sigmoid curve. The dose corresponding to 50 per cent mitotic inhibition after 6 hours is $315 \begin{smallmatrix} + 25 \\ - 22 \end{smallmatrix}$ r.

The mitotic inhibition produced by Compound I in the chick fibroblast cultures has been studied in some detail after 24 hours' application. The results are summarised in Fig. 2, in which the mitotic inhibition in probits is plotted against the logarithm of the molar concentration. The calculated regression line gives a satisfactory fit. There is no indication of departure from a linear relation between mitotic inhibition in probits and the logarithm of the concentration. Thus there is a sigmoid relation between mitotic inhibition and the applied concentration. For 50 per cent mitotic inhibition after 24 hours the concentration of I is $3{\cdot}81 \pm 0{\cdot}15 \times 10^{-6}$ molar.

The calculation of the regression line is somewhat laborious. For most of the compounds studied such accuracy is not necessary. It is justifiable to deduce the approximate concentration required to give 50 per cent mitotic inhibition from the curve fitted by eye to relate the observed percentage mitotic inhibition to the molar concentration plotted on a log scale. In the case of Compound I this method gave a value of $3{\cdot}84 \times 10^{-6}$M.

The first experiments on the antimitotic effects of X-rays and I in chick fibroblast cultures (Mitchell and Simon-Reuss, 1947) showed much greater mitotic inhibition with the combination of 3×10^{-6}M compound and 244 r of X-radiation delivered 18 hours later, with fixation and counting after a further period of 6 hours, than with the same amounts of the two agents separately.

To test for potentiation rigorously, it is essential to compare the effect of a combination of half amounts (or other suitable mixtures) of the two agents with the mean effect of the agents acting independently.

This method, which may be termed the summation method, is well recognised in pharmacology, and has already been applied in radiobiology by Liechti and Muller (1936), Gray and Read (1944), and Mitchell (1947).

Our original experiments showed a combined effect of 86·7 per cent mitotic inhibition, while the corresponding mean calculated for the two agents separately from the regression lines in Fig. 1 and 2, is 78·6 per cent. This result suggests that there is at least additivity of the effects, but it does not provide evidence for potentiation.

In other experiments, the combined effect of 2×10^{-6}M compound followed

by 400 r of X-radiation delivered in 2.00 minutes with fixation 6 hours later produced 95·6 per cent mitotic inhibition. The compound alone at 4×10^{-6}M produces 54·4 per cent mitotic inhibition and 800 r alone 88·3 per cent mitotic inhibition, so that the mean has the value 71·4 per cent. This result is strongly suggestive of potentiation.

Two special series of experiments were carried out to study potentiation of the antimitotic effect of Compound I and X-radiation in the chick fibroblast cultures. In these experiments the X-radiation was delivered at 200 r per minute to reduce errors in dosage. Details of one set of experiments are given in Table I.

In the other sets of experiments 4×10^{-6} M compound produced 38·4 per cent mitotic inhibition after 24 hours, and 400 r of X-radiation produced 59·5 per cent mitotic inhibition after 6 hours ; the mean is 48·95 per cent. The combination of 2×10^{-6}M compound followed after 18 hours by 200 r of X-radiation produced 93·25 per cent mitotic inhibition after 24 hours. From the number of mitoses in the individual cultures it is found that for 8 degrees of freedom Student's t is 15·71, so that P is much less than ·001.

In this experiment, as in Table I, the highest values of the mitotic inhibition were associated with no significant reduction of the area of the outgrowth. This finding confirms the unimportance of cell migration under the experimental conditions of the combined action of X-rays and the Compound I.

The possibility of differences in the behaviour of the outgrowth and of the central part of the culture has been studied by means of the counts made in serial sections of 24-hour cultures. The method is a laborious one ; by its use, Fischer and Parker (1929) showed that in chick periosteal fibroblasts, the proportion of cells in mitosis was 0·79 per cent in the outgrowth, but only about 0·09 per cent in the central part. In our cultures—as in those of Jacoby (1937)—the mitoses in the central part are limited to the " perichondrium " on its surfaces. Studies of the topographical distribution of mitoses in our cultures by Dr. A. F. Phillips showed no appreciable decrease in the number of cells in mitosis per unit area as the central part is approached. The area of the central part is usually not more than 20 per cent of the total area of the culture. In the serial sections of the cultures the central part is easily defined. The resting and mitotic cells were counted in the surface layers. In the sections of a control culture, out of 6205 cells counted there were 53 cells in mitosis, of which 13 were in prophase, 31 in metaphase, 3 in anaphase and 6 in telophase. At 24 hours after the application of 5×10^{-6}M Compound I, out of 6220 cells counted, there were 26 cells in mitosis ; of these 1 was in prophase, 19 were in metaphase, none were in anaphase and 6 were in telophase. By the use of a statistical method (Haldane, 1945), it can be shown that the ratio of the frequency of mitosis in the central part of the treated culture to that in the control is $0 \cdot 478 \pm 0 \cdot 118$. The corresponding value for the outgrowth as determined by the exact method (Fig. 2) is $0 \cdot 258 \begin{smallmatrix} + \cdot 040. \\ - \cdot 038. \end{smallmatrix}$ The experimental error in the value for central part of the culture is considerable. The difference between the ratio of the mitotic frequency of the treated and control cultures for the outgrowth and the central part ($0 \cdot 220 \pm 0 \cdot 124$) is not significant.

For the experimental conditions it is concluded that the combination of the action of X-radiation and of the Compound I shows potentiation of mitotic inhibition, and that the mechanism of action of the two agents is different.

TABLE I.—*Combination of Effects of Compound I and X-radiation in Chick Fibroblast Cultures.*

Treatment.	Time of fixation after treatment. (hours.)	Number of cultures.	Number of mitoses in individual cultures.						Total number of cells in mitosis.	Per cent mitotic inhibition.	Mean area of outgrowth per culture × (27·5)²cm.²	Per cent reduction of area of outgrowth.
a. Control	24	6*	160	131	138	169	176	158	932	—	63·2	—
b. 2×10^{-6}M Compound I	24	6	96	121	105	108	107	114	651	30·15	53·6	15·2 ± 8·1
c. 4×10^{-6}M Compound I	24	6	92	79	88	90	84	78	511	45·18	48·0	24·0 ± 7·9
d. 150 r X-radiation	6	6	108	114	111	127	120	136	716	23·19	52·8	16·5 ± 7·9
e. 300 r X-radiation	6	6*	71	105	101	88	90	84	539	42·16	44·0	30·4 ± 6·0
f. 2×10^{-6}M Compound I, followed after 18 hr. by 150 r X-radiation	24	6*	16	35	24	18	27	31	151	83·80	57·7	8·7 ± 8·3
g. 4×10^{-6}M Compound I, followed after 18 hr. by 300 r X-radiation	24	6*	8	5	16	10	9	7	55	94·1	64·9	− 2·7 ± 8·8

* 5 cultures only for measurement of area of outgrowth.

The mean of the effects of 4×10^{-6}M compound (*e*) and 300 r X-radiation (*e*) is compared with the effect of the combination (*f*) of half quantities of the two agents. The mean mitotic inhibition in (*c*) and (*e*) is 43·67 per cent while the mitotic inhibition in (*f*) is 83·80 per cent. The mean for (*c*) and (*e*) calculated from the regression lines in Fig. 1 and 2 is 50·6 per cent.

Considering the numbers of mitoses in the individual cultures using means of paired cultures in (*c*) and (*e*) and the observed numbers in (*f*), for 10 degrees of freedom, Student's t is 14·64 and P is much less than ·001. Hence the mitotic inhibition in (*f*) is significantly higher than the mean mitotic inhibition in (*c*) and (*e*) and potentiation is confirmed.

The measurement of the area of the outgrowth is subject to considerable experimental error, including systematic error which is not shown in the Table. However, it is clear that the highest values of the mitotic inhibition observed in (*f*) and (*g*) are not associated with significant reduction of the area of the outgrowth.

Cytological Effects.

The cytological changes produced by Compound I in the chick fibroblast cultures differ in some ways from those produced by X-rays. Perhaps the most fundamental difference is that anaphase bridges are only rarely produced by the compound, unlike X-rays.

After the application of Compound I to the living cells, the first change observed is temporary cell enlargement, presumably due at least in part to hydration (" cell oedema "). This does not occur with 5×10^{-7}M concentration, but has been observed in its early stages at 2 minutes after application of the compound in concentration 5×10^{-6}M. The change is obvious after 5 minutes and probably affects the cytoplasm first. After 10 minutes the effects are still increasing ; there is bubbling of the enlarged cytoplasm and enlargement of the mitochondria, nucleus and nucleolus. The changes reach a maximum after about 20 minutes and then very slowly decrease. After 24 hours only some of the dividing cells are still enlarged. At these later hours it is difficult to attribute the enlargement of the dividing cells to hydration.

Examples of these changes are shown in Fig. 3, 4 and 5. The slight transient shrinkage of the cell immediately after addition of the compound is observed consistently.

At the maximum of the cell enlargement the increase in transverse dimensions of the cells is often in the region of 50 per cent.

Mitotic inhibition is obvious after 80 minutes and persists after 24 and 36 hours. Counts show that there is no appreciable change in the distribution of the phases of mitosis even in high concentrations such as 5×10^{-5}M where mitotic inhibition is almost complete. There are relatively few abnormal mitoses. For example at 4×10^{-6}M concentration the abnormal mitoses increased slowly from about 5 per cent of the total mitotic count after 80 minutes to about 15 per cent after 24 hours. A typical count of the mitotic abnormalities is included in Table II, and photomicrographs of examples of the abnormal cells after fixation are shown in Fig. 6, 7, 8, 9 and 10. Enlargement of some of the dividing cells is characteristic (Fig. 6, 7 and 9). The commonest abnormality is clumping of the chromosomes, usually observed at metaphase (Fig. 8). Although it is difficult to study the chromosomes in the chick fibroblasts in culture, it is possible to discern chromosome fragmentation and chromosomes showing regions of impaired stainability, presumably due to deficiency of deoxyribonucleic acid, and irregular and " beaded " structure, similar to the " erosion " described by Levan (1949) (Fig. 7). Multipolar mitoses are not very common (Fig. 9). Anaphase bridges are almost never produced by the compound alone, but typical anaphase bridges are observed after the combined action of the compound and X-radiation (Fig. 10).

At high concentrations, e.g., at 24 hours after the application of 5×10^{-5}M concentration, when only few mitoses persist, many of the resting cells are flattened and still enlarged, and most of the metaphases are clumped and enlarged with cytoplasmic vacuolation. The outgrowth is reduced and irregular.

The mitotic abnormalities produced by the combined action of Compound I and X-radiation are exemplified in Table II. With the combination (*f*), clumped metaphases are the commonest mitotic abnormality, but chromosome fragmentation occurs with a frequency convincingly higher than that to be expected on the basis of additivity of the effects in (*c*) and (*e*) ; $\chi^2 > 16{\cdot}9$ for 1 degree of freedom, so that P is less than ·001. It is concluded that the combined action of Compound

TABLE II.—*Abnormal Mitosis Produced by Combination of Effects of Compound I and X-radiation in Chick Fibroblast Cultures.*

(Same experiment as in Table I.)

Treatment.	Time of fixation after treatment (hours)	Number of cultures.	Total number of cells in mitosis	Total number of cells with abnormal mitosis.	Mitotic abnormalities.				
					Binucleate prophase	Clumped metaphase	Chromosome fragmentation—observed only at metaphase.	Cells with anaphase bridges.	Telophase with undivided cytoplasm.
e. 4×10^{-6}M Compound I	24	6	511	78	6	51	9	—	12
e. 300 r X-radiation	6	6	539	119	3	84*	23	6	2
f. 2×10^{-6}M Compound I Followed after 18 hr. by 150 r X-radiation	24	5	120	52	—	23	17	—	12

* Also 1 tripolar metaphase.

I and X-radiation shows potentiation of chromosome fragmentation. This finding is of particular interest in connection with possible radiotherapeutic applications.

Instability of Compound I in Solution.

Studies of the thermal instability of Compound I in aqueous solution were made because of erratic results in animal experiments and clinical trials. Compound I in solutions containing 500 mg. in 5 ml. in nitrogen-filled ampoules, as supplied by the manufacturers, was incubated at 39° C. for varying intervals, and then applied to the chick fibroblast cultures in concentration 5×10^{-6}M for 24 hours in the usual way. The mitotic counts obtained are given in Table III. It

TABLE III.—*Instability of Compound I.*

Mitotic counts in cultures fixed at 24 hours after application of 5×10^{-6}M. Compound I which had been incubated at 39°C. for varying intervals in solutions containing 500 mg. in 5 ml. in nitrogen-filled ampoules.

The fresh solution produces $74{\cdot}3 \begin{matrix} + 4{\cdot}1 \\ - 3{\cdot}7 \end{matrix}$ per cent mitotic inhibition.

Treatment.	Days of incubation.	Numbers of cultures.	Total number of cells in mitosis.	Number of cells in				Per cent mitotic inhibition.
				Prophase.	Metaphase.	Anaphase.	Telophase.	
Control	—	6	981	168	319	47	447	—
Incubated	1	5	464	76	209	30	149	$43{\cdot}3 \pm 7{\cdot}7$
,,	2	6	806	116	388	66	236	$17{\cdot}8 \pm 10{\cdot}3$
,,	4	5	141	15	96	7	23	(83 ± 39)
Control	—	6	990	124	382	86	398	—
Incubated	7	6	897	217	307	37	336	$9{\cdot}4 \pm 7{\cdot}0$

has been shown that the fresh untreated solution under similar conditions produces $74{\cdot}3 \begin{matrix} + 4{\cdot}1 \\ - 3{\cdot}7 \end{matrix}$ per cent mitotic inhibition.

Incubation at 39° C. for 7 days almost completely destroys the antimitotic activity and produces a rather toxic solution. There is no significant mitotic inhibition, but the outgrowth is poor, many of the resting cells are vacuolated, disconnected and rounded and a large number of the cells in metaphase show gross chromosome clumping. There is progressive reduction of the mitotic inhibition

observed after incubation for 1 and 2 days. After 4 days' incubation the solution is toxic. The outgrowth is poor, many of the resting cells are rounded, there is some accumulation in metaphase and a number of exploded cells; there is wide variation in the behaviour of different cultures. It seems likely that there are several unstable intermediate products involved in the process of inactivation of Compound I by incubation at 39° C. in nitrogen-filled ampoules. It may be mentioned that the incubated solutions showed no colour change.

DISCUSSION.

The use of the technique of tissue cultures makes it possible to examine quantitatively the effects of many chemical agents on a particular type of living cell using amounts of the compounds in the region of milligrams. The method is useful as a sorting test for chemical agents of possible therapeutic interest but its limitations to the type of cell, species and defined experimental conditions must be emphasized. Almost invariably the information obtained is preliminary to animal experiments.

Tetra-sodium 2-methyl-1:4-naphthohydroquinone diphosphate (Compound I) has been studied in detail as a reference substance. It is found that there is no indication of departure from a linear relation between mitotic inhibition in probits and the logarithm of the concentration of the compound. Accordingly it is suggested that the best estimate of the activity of the compound is the concentration which produces 50 per cent mitotic inhibition under the experimental conditions. This concentration may be termed MI 50 as a convenient abbreviation. Among the related compounds studied (Mitchell and Simon-Reuss, 1952) most show a similar behaviour. An anomalous mitosis-concentration relation indicates mechanisms of biological action different from the quantal (all-or-nothing) response involved in the blockage of the entry of cells into mitosis. For example, a rapidly increasing prolongation of metaphase with concentration, at high concentrations of a compound may lead to a maximum of apparent mitotic inhibition; this appears to occur in the case of Compound VIII (Mitchell and Simon-Reuss, 1952). It is of course necessary to study the mitotic phase distribution and cytological effects with any compound examined.

It is of great interest that with small doses of X-radiation under the present experimental conditions there is also no indication of departure from a linear relation between mitotic inhibition in probits and the logarithm of the dose of radiation.

The quantitative consideration of potentiation in the study of the effects of combinations of agents is a difficult problem of general biological importance. For the present purpose of selecting chemical agents as possible therapeutic radiosensitisers the summation method as applied here to the joint action of Compound I and small doses of X-radiation appears to be adequate and sufficiently simple for routine testing.

The first step is to select a compound which is a mitotic inhibitor, and with which the combined action of X-radiation shows potentiation of mitotic inhibition. However, mitotic inhibition is not the only factor involved, and probably it is not the most important factor to be considered. Studies of the mechanism of the therapeutic action of radiations and investigations of possible chemotherapeutic agents suggest that mitotic inhibition *per se* is not directly responsible

for the retrogression of malignant tumours. There is much radiobiological evidence to support the view that the most important mechanism by which ionising radiations kill cells is chromosome breakage and reunion, with loss of chromosome parts and the production of inviable structural aberrations of the chromosomes. The relation between chromosome damage by radiation and the disturbance of the cellular metabolic processes is not understood. The role of chromosome structural changes in radiotherapy is, at present, uncertain, However, it seems plausible to expect potentiation of chromosome fragmentation in the combined action of X-radiation and of a chemical agent which may be a therapeutic radiosensitiser. This property is shown by Compound I. It seems reasonable to try to improve upon this compound by selecting compounds of related chemical structure, which alone produce more chromosome fragmentation, preferably together with anaphase bridges.

SUMMARY.

1. More satisfactory quantitative experimental methods using the technique of tissue culture have been developed to study mitotic inhibition and radiosensitisation by chemical agents, using tetrasodium 2-methyl-1:4-naphthohydroquinone diphosphate (Compound I) as the reference substance.

2. The best estimate of the antimitotic activity of the compound is the concentration which produces 50 per cent mitotic inhibition under the experimental conditions. Studies of cytological effects are even more important than those of mitotic inhibition. Cell migration has been shown to be unimportant in these experiments.

3. For Compound I, there is no indication of departure from a linear relation between the mitotic inhibition in probits and the logarithm of the concentration.

For 50 per cent mitotic inhibition after 24 hours, the concentration is $3{\cdot}81 \pm 0{\cdot}15 \times 10^{-6}$M.

4. For X-radiation there is no indication of departure from a linear relation between mitotic inhibition in probits and the logarithm of the dose.

5. The study of the combination of the action of X-radiation and Compound I by means of the summation method has demonstrated potentiation of both mitotic inhibition and chromosome fragmentation.

6. Thermal instability of Compound I in aqueous solution in the absence of oxygen has been demonstrated. There is almost complete inactivation after incubation at 39° C. for 7 days. This finding may account for some erratic results in animal experiments and clinical trials.

We are indebted to Dr. F. Wrigley of Roche Products, Limited, Welwyn Garden City, for arranging supplies of "Synkavit".

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EXPERIMENTS ON THE MECHANISM OF ACTION OF TETRA-SODIUM 2 - METHYL - 1 : 4 - NAPHTHOHYDROQUINONE DIPHOSPHATE AS A MITOTIC INHIBITOR AND RADIOSENSITISER, USING THE TECHNIQUE OF TISSUE CULTURE: RELATION BETWEEN CYTOLOGICAL EFFECTS AND CHEMICAL CONSTITUTION.

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Received for publication June 3, 1952.

THE study of the biological effects on chick fibroblast cultures has been used as the main sorting test for possible therapeutic radiosensitisers. It seems reasonable to select a compound which not only blocks the entry of cells into mitosis but also produces chromosome breakage, preferably together with anaphase bridges. Other desirable properties are low toxicity, stability and a molecular structure suggesting ease of penetration into cells. It seems probable that a colchicine-like action is not essential for radio-sensitisation. There appears to be no conclusive evidence that colchicine itself is of any practical value as a radiosensitiser (Levine, 1951).

Since 1946 an attempt has been made in collaboration with Dr. E. J. Friedmann and Dr. D. H. Marrian of this Department to study antimitotic action in relation to chemical structure. In all, about 100 compounds have been tested on chick fibroblast cultures. In addition to the reports by Mitchell (1949, 1950, 1951), a number of papers dealing mainly with mitotic inhibition have been published on the following groups of compounds and aspects of the problem: certain quinones (Friedmann, Marrian and Simon-Reuss, 1948*a*), sulphydryl addition compounds of some quinones and related compounds (Friedmann, Marrian and Simon-Reuss, 1948b), maleimide and related substances (Friedmann, Marrian and Simon-Reuss, 1949), the reactions of substituted maleimides with thiols (Marrian, 1949*a*), the condensation of N-substituted maleimides with thiourea (Marrian, 1949*b*), the action of 1:4-naphthohydroquinone diphosphate (Friedmann and Bailey, 1950), unsaturated imides with special reference to their reaction with sulphdryl groups (Friedmann, Marrian and Simon-Reuss, 1952*a*), halogen derivatives of the 1:4-naphthoquinone group and the maleic acid series (Friedmann, Marrian and Simon-Reuss, 1952*b*), and a spectrometric investigation of the interation of glutathione with maleimide and N-ethylmaleimide (Friedmann, 1952). As a different method of approach, studies are in progress on the antagonisation of the antimitotic action of tetra-sodium 2-methyl-1:4-naphthohydroquinone diphosphate (Compound I) by nucleotides, nucleosides, purines and pyrimidines and some other compounds (Mitchell, 1950, 1951).

This paper is an account of the cytological effects of a series of 29 selected

compounds allied to tetra-sodium 2-methyl-1:4-naphthohydroquinone diphosphate (Compound I) from the points of view of—

(a) the relation of the chemical constitution to the biological action of Compound I, and

(b) the selection of new compounds as possible therapeutic radiosensitisers.

Although many of these compounds have been described previously from the point of view of mitotic inhibition and their preparation and chemical properties, little has been published about their cytological effects and new information is given about 12 compounds which we have examined.

EXPERIMENTAL METHODS.

The experimental methods are those described by Mitchell and Simon-Reuss (1952). In the case of a few compounds of interest, experiments have been made to test for radiosensitisation in the tissue cultures, using the summation method.

Recently some water-insoluble compounds have been tested in solution in ethylene glycol mono-ethyl ether (Cellosolve) combined with gum Ghatti, using the method proposed by Friedmann and Simon-Reuss (1951). However, we have found that these agents in the concentrations used produce a significant increase in the mitotic inhibition observed at 6 hours after 300 r of X-radiation, so that caution is necessary in the application of this method to studies of radiosensitisation.

Preparations used.

Most of the chemicals used in this investigation were either prepared or purified by Dr. E. J. Friedmann and Dr. D. H. Marrian. Details of preparations have been published as follows: Compounds II and IV (Friedmann, Marrian and Simon-Reuss, 1948*a*), Compounds XX–XXIII (Friedmann, Marrian and Simon-Reuss, 1948*b*), Compounds VIII, IX and XXIV (Friedmann, Marrian and Simon-Reuss 1952*a*).

We are indebted to Dr. F. Bergel, of Roche Products, Limited, for the following compounds and information about them: Compound X, $C_{11}H_5O_{10}P_2Na_5 . 4C_2H_5OH . 5H_2O$, mol. wt. 748·2, calc.: C, 30·5; H, 5·2; P, 8·3; found: C, 30·52; H, 3·1; P, 8·33, 8·20; Compound XI, $C_{12}H_4O_{12}P_2Na_6 . 7H_2O$, mol. wt. 666·2, calc.: C, 21·7; H, 2·7; P, 9·3; found: C, 21·9; H, 3·3; P, 9·2, 9·4; Compound XIV, $C_9H_{10}O_8P_2Ca_2 . H_2O . \frac{1}{2} C_2H_5OH$, mol. wt. 429·41, calc.: C, 28·0; H, 3·5; P, 14·45, found: C, 28·67; H, 3·27; P, 14·37, 14·10. The specimen of vitamin K_1 was kindly given by Dr. F. Wrigley of Roche Products, Limited.

Compound XXVII, described by Tarbell, Fukushima and Dam (1945) and Kitchen and Sandin (1945) was prepared by Dr. Friedmann.

Compound XXVIII was prepared by Dr. Marrian, who has kindly given us the following information about the di-barium salt of 2:3-dimethyl-1:4-naphthohydroquinone diphosphate: Colourless prisms, $C_{12}H_{10}O_8P_2Ba_2 . 5H_2O$, calc.: C, 20·4; H, 2·82; P, 8·75; loss of $5H_2O$, 12·7 per cent; found: C, 20·0; H, 2·72; P. 8·55; loss at 120° 11·6 per cent; at pH2 the molecular extinction coefficient has the values $5·42 \times 10^3$ at 290 mμ. and $6·24 \times 10^4$ at 230 mμ.

TABLE I.—*Effects of some Chemical Agents on Chick Fibroblasts in Tissue Culture.*

Hanging drop technique, fourth passage, fixed after 24 hours' incubation at 39°C.

Number.	Compound.	Molar concentration to produce 50 per cent mitotic inhibition.	Summary of cytological effects.
I	2-methyl-1:4-naphthohydroquinone diphosphate, tetra-sodium salt	$3{\cdot}81 \pm 0{\cdot}15 \times 10^{-6}$	Reference substance (Mitchell and Simon-Reuss, 1952). No change in mitotic phase distribution even at 5×10^{-5}M. Temporary cell enlargement in early hours. Few abnormal mitoses. At 4×10^{-6}M, 15 per cent of mitoses abnormal, mainly clumped metaphases with some chromosome fragmentation and chromosomes showing regions of impaired stainability and irregular and beaded structure, binucleate prophases and undivided telophases. Practically no anaphase bridges.
II	1:4-naphthohydroquinone diphosphate, tetra-sodium salt	$3{\cdot}0 \times 10^{-9}$	Temporary cell enlargement in early hours. At 3×10^{-9}M, 15 per cent of mitoses abnormal. At 1×10^{-8}M, 45 per cent of mitoses abnormal, mainly clumped metaphases with some chromosome fragmentation and some multipolar cells. Increasing metaphase accumulation from 35 per cent of mitoses at 5×10^{-9}M to 85 per cent at 5×10^{-7} and 1×10^{-6}M.
III	2-methyl-1:4-naphthohydroquinone disuccinate	Probably inactive	Unstable. Less than 15 per cent mitotic inhibition at 6×10^{-6}M. Resting cells vacuolated, and disconnected at 9×10^{-6}M. No change in phase distribution.
IV	1:4-naphthohydroquinone monohydrogen succinate	Inactive in concentrations tested	No mitotic inhibition or cytological changes at $1–5 \times 10^{-9}$M.
V	2-methyl-1:4-naphthoquinone	$\sim 2 \times 10^{-5}$ (extrapolated)	Tested in solution in Cellosolve and gum Ghatti. Little mitotic inhibition at $1–5 \times 10^{-6}$M. Some clumped metaphases. At 1×10^{-5}M, 32 per cent mitotic inhibition and toxic effect on resting cells which increases rapidly with concentration.
VI	Phthiocol, 2-methyl-3-hydroxy-1:4-naphthoquinone	4×10^{-6}	Tested in Cellosolve and gum Ghatti. At 4×10^{-6}M, clumped metaphases with some chromosome fragmentation and many undivided telophases. Metaphase accumulation starting at 1×10^{-5}M.
VII	2-methyl-1:4-naphthoquinone-2:3-oxide	Inactive for mitotic inhibition	Tested in Cellosolve and gum Ghatti. No mitotic inhibition at $10^{-6}–10^{-5}$M. Some clumped metaphases at 1×10^{-5}M.

TABLE I—(*cont.*).

Number.	Compound.	Molar concentration to produce 50 per cent mitotic inhibition.	Summary of cytological effects.
VIII	2-bromo-1:4-naphthohydroquinone diphosphate, tetra-sodium salt	$\sim 4 \times 10^{-6}$	The mitosis-concentration relation is anomalous with a minimum in the region of 50 per cent due to metaphase accumulation at higher concentrations. Abnormal cells are found in concentrations 1–8 $\times 10^{-6}$M, mainly cell enlargement, chromosome fragmentation, binucleate prophases, multipolar and undivided telophases. The spindle is well defined in some cells. At the highest concentrations are many exploded cells, and rounded and disconnected resting cells. Appearances suggest toxicity.
IX	2-methyl-3-bromo-1:4-naphthohydroquinone diphosphate, tetra-sodium salt	$\sim 7 \times 10^{-6}$	Abnormal mitoses, mainly clumped metaphases and chromosome fragmentation. Some metaphase chromosomes show areas of impaired stainability. Practically no anaphase bridges. Metaphase accumulation with some spindle abnormalities. The same types of cytological abnormalities are found in all concentrations examined but abnormalities are most frequent in the lowest concentration 1×10^{-6}M, which is the only concentration showing appreciable metaphase accumulation. Phase distribution is within normal limits at higher concentrations. (Fig. 2 to 5.)
X	1:4-dihydroxy-2-naphthoic acid diphosphate, penta-sodium salt	5×10^{-6}	Very few abnormal mitoses. At 8×10^{-6}M, slight metaphase accumulation and some rounding up and vacuolisation of resting cells. Practically no anaphase bridges.
XI	1:4-dihydroxy-2:3-naphthalene dicarboxylic acid diphosphate, hexa-sodium salt	9×10^{-6}	Abnormal mitoses in all concentrations examined from 1–10 $\times 10^{-6}$M, and increasing with concentration. Mainly clumped metaphases and anaphases, and chromosome fragmentation with some anaphase bridges. Some metaphase chromosomes show regions of impaired stainability. Slight metaphase accumulation and some spindle abnormalities at the higher concentrations. At 10^{-5}M there are many exploded and degenerating cells, and many resting cells are rounded up and vacuolated. (Fig. 6 to 9.)
XII	Toluhydroquinone	$\sim 5 \times 10^{-5}$ (approximate value only)	At 1×10^{-5}M no detectable mitotic inhibition but mitotic abnormalities, mainly clumped prophases, with chromosome fragmentation, a few anaphase bridges and some multipolar mitoses. At 5×10^{-5}M accumulation of cells in metaphase was shown by recording film technique.
XIII	Toluhydroquinone diphosphate, tetra-sodium salt	$\sim 1 \times 10^{-5}$	Some metaphase accumulation, with clumped metaphases, chromosome fragmentation and multipolar mitoses.

TABLE I—(*cont.*).

Number.	Compound.	Molar concentration to produce 50 per cent mitotic inhibition.	Summary of cytological effects.
XIV	2:3:6-trimethylhydroquinone diphosphate, di-calcium salt	5×10^{-6}	In concentrations higher than 1×10^{-6}M many abnormal mitoses, especially clumped metaphases and anaphases, some chromosome fragmentation, some very large dividing cells and a few tri- and multipolar cells. At 8×10^{-6}M many rounded-up and disconnected resting cells. No change in phase distribution. (Fig. 10.)
XV	Hydroquinone	6×10^{-9}	Slow increase of mitotic inhibition with increasing concentration. No change in phase distribution. Few abnormal mitoses, mainly clumped metaphases.
XVI	Hydroquinone diphosphate, tetra-sodium salt	3×10^{-9}	Much more rapid increase of mitotic inhibition with increasing concentration. At 8×10^{-9}M few prophases and increase in anaphases. Very few abnormal mitoses, these mainly clumped metaphases.
XVII	Phenol	1×10^{-5}	Slight metaphase increase in lower concentrations. Some clumped metaphases.
XVIII	Phenyl phosphate, di-sodium salt	$\sim 5 \times 10^{-7}$	Slow increase in mitotic inhibition with increasing concentration. No change in phase distribution. Enlargement of some resting and dividing cells, and some clumped metaphases.
XIX	p-nitrophenyl phosphate, di-sodium salt	$\sim 5 \times 10^{-6}$	In this region of concentration there are many abnormal mitoses, mainly clumped metaphases and anaphases and multipolar cells, together with many enlarged, vacuolated and disconnected resting cells, and many exploded cells. There is slight metaphase accumulation at higher concentrations. Appearances suggest toxicity.
XX	S-(2-methyl-1:4-naphthoquinonyl-3-) glutathione	2×10^{-6}	Mitosis-concentration relation is anomalous, maximum mitotic inhibition reached being about 55 per cent. Slight metaphase accumulation at the lower concentrations but normal phase distribution in region of maximum mitotic inhibition. Some mitotic abnormalities, mainly clumped metaphases at all concentrations examined.
XXI	S-(2-methyl-1:4-naphthoquinonyl-3-) thiolacetic acid	Inactive	In concentrations $1–6 \times 10^{-6}$M no mitotic inhibition and no cytological abnormalities; phase distribution normal.
XXII	S-(1:4-naphthoquinonyl-2-) glutathione	,,	
XXIII	S-(1-4-naphthoquinonyl-2-) thiolacetic acid	,,	

TABLE I—(*cont.*).

Number.	Compound.	Molar concentration to produce 50 per cent mitotic inhibition.	Summary of cytological effects.
XXIV	S-(2-chloro-1:4-naphtho-quinonyl-3-) glutathione	Inactive	In concentrations 1–5 $\times$ 10^{-6}M no mitotic inhibition and no cytological abnormalities; phase distribution normal.
XXV	Mapharside (mapharsen)	Inactive for mitotic inhibition	No mitotic inhibition and no change in phase distribution but rather toxic. At 5 $\times$ 10^{-6}M many resting cells rounded-up and processes retracted.
XXVI	Neoarsphenamine	Inactive for mitotic inhibition	At 3 $\times$ 10^{-6}M no change in phase distribution but a few clumped metaphases and anaphases, and some rounding-up of resting cells. At 5 $\times$ 10^{-6}M mitoses still present but not countable because of toxic effect on resting cells, almost all of which are rounded and disconnected. All recognisable metaphases are clumped.
XXVII	5-methyl-4:7-thionaphthene quinone	$\sim 5 \times 10^{-6}$	At 5 $\times$ 10^{-6}M slight cell enlargement and some clumped metaphases. At 1 $\times$ 10^{-5}M metaphase accumulation, with 65 per cent of mitotic cells in metaphase.
XXVIII	2:3-dimethyl-1:4-naphtho-hydroquinone diphosphate, tetrasodium salt	7×10^{-6}	Metaphase accumulation and spindle abnormalities even at 2 $\times$ 10^{-6}M, but not at 5 $\times$ 10^{-7}M. At 1·2 $\times$ 10^{-5}M no cells were seen in prophase and about 60 per cent of mitotic cells were in metaphase, while the resting cells were flattened and disconnected and the outgrowth was slight and irregular. In addition to metaphases with gross chromosome clumping and arrested metaphases with cytoplasmic vacuolation, characteristic appearances are anaphase bridges, chromosome fragmentation and chromosomes with regions of impaired stainability. (Fig. 11 to 18.)
XXIX	Vitamin K_1, 2-methyl-3-phytyl-1:4-naphthoquinone	Inactive for mitotic inhibition	Tested in Cellosolve and gum Ghatti. At 5 $\times$ 10^{-6}M no mitotic inhibition and no appreciable change in mitotic phase distribution but some spindle abnormalities with anaphase bridges and chromosome fragmentation and a number of telophases with undivided cytoplasm. At 1 $\times$ 10^{-5}M many resting cells are flattened and disconnected. (Fig. 19, 20.)
XXX	2:4-dinitrophenol	5×10^{-6}	At this concentration many enlarged cells and clumped metaphases with some chromosome fragmentation, also some undivided telophases. With increasing concentration at 1–5 $\times$ 10^{-5}M slowly increasing mitotic inhibition with increasing metaphase accumulation and toxic effect on resting cells. At 5 $\times$ 10^{-5}M most metaphases clumped, most resting cells flattened, rounded up and disconnected and some exploded cells.

RESULTS.

The effects of the selected chemical agents on the chick fibroblasts in culture are summarised in Table I. The cytological effects are considered in relation to those of Compound I. Of especial interest from the point of view of the selection of compounds as possible therapeutic radiosensitisers is the appearance of mitotic abnormalities in concentrations which produce mitotic inhibition without gross toxic effects on the resting cells. The most important abnormalities studied are chromosome fragmentation, anaphase bridges, metaphase chromosomes with regions of impaired stainability and with irregular and beaded structure, metaphases with clumping of the chromosomes, arrested metaphases and other spindle abnormalities, of which many of the types described by Barber and Callan (1943) have been noted. As already discussed (Mitchell and Simon-Reuss, 1952), it seems likely that the most relevant cytological abnormalities are chromosome fragmentation, anaphase bridges and chromosome aberrations in general. Examples of the various types of cytological effects observed with this series of compounds are shown in Fig. 2 to 20. It is evident that none of the abnormalities are specific for particular compounds, but in the figures an attempt is made to select the commonest or most characteristic cytological changes associated with the more interesting compounds.

The formulae of the compounds are given in Table II. These "pictorial" representations of the chemical structure have been supplemented by studies of more accurate molecular models of the "space filling" type (Hartley and Robinson, 1952), e.g., Fig. 1 shows photographs of models of the molecule of Compound XXVIII.

Discussion of Cytological Action in Relation to Chemical Constitution.

The main factors to be considered are :

(1) the importance of phosphorylation,
(2) the influence of the methyl group,
(3) the role of the carboxyl group,
(4) the influence of other groups especially bromine, hydroxyl and oxide,
(5) alteration of the ring structure,
(6) interactions with sulphydryl groups, and
(7) comparison with vitamin K activity.

(1) *Phosphate groups.*

Phosphorylation almost invariably increases the antimitotic activity.

2-Methyl-1:4-naphthoquinone (V) produces no detectable mitotic inhibition in concentration 1×10^{-6}M, about 7 per cent mitotic inhibition in 5×10^{-6}M and 32 per cent mitotic inhibition in 1×10^{-5}M when tested in solution in Cellosolve and gum Ghatti, the presence of which does not affect the Compound I. There are toxic effects on the resting cells at 1×10^{-5}M concentration and these increase rapidly with increasing concentration, so that it is impossible to determine directly the concentration corresponding to 50 per cent mitotic inhibition (MI50) for this compound. A specimen of 2-methyl-1:4-naphthohydroquinone, which is a poorly characterised compound with no sharp melting-point (Fieser, 1941, p. 235), also produced no significant mitotic inhibition in concentration 10^{-6}–10^{-5}M when tested in Cellosolve and gum Ghatti. Thus phos-

phorylation increases the antimitotic activity under the experimental conditions by a factor of at least 5. Moreover, replacement of the two phosphate groups in Compound I by two succinate groups leads to Compound III which is unstable but almost certainly of very low activity. Similarly in relation to Compound II, 1:4-naphthohydroquinone monohydrogen succinate (IV) is of low activity. It is possible, though unlikely, that the cultures and the medium lack enzymes capable of hydrolysing the succinates.

EXPLANATION OF PLATES.

FIG. 1.—Photograph of model of molecule of tetra-sodium 2:3-dimethyl-1:4-naphthohydroquinone diphosphate (Compound XXVIII).

The atomic models are of the "space filling" type (Hartley and Robinson, 1952). The naphthalene ring is viewed laterally. Nearest the scale are the two methyl groups in the 2- and 3- positions. The phosphoric ester groups are seen laterally in the 1- and 4- positions. The P atoms are largely covered by the O atoms (white). The centres of the most lateral O atoms, which are covered by the small grey H atoms, are separated by a distance which lies between 9·5 and 10·5 Å.

FIG. 2 to 5.—Effect of tetra-sodium salt of 2-methyl-3-bromo-1:4-naphthohydroquinone diphosphate (Compound IX) on chick fibroblast cultures. Concentration 5×10^{-6}M. Fixed after 24 hours. × 1200.

FIG. 2.—Arrested metaphases. Upper cell shows a chromosome with regions of impaired stainability.

FIG. 3.—Metaphase with gross clumping of chromosomes.

FIG. 4.—Anaphase with irregular distribution of chromosomes.

FIG. 5.—Anaphase with incompact spindle and chromosome bridges.

FIG. 6 to 9.—Effects of hexa-sodium salt of 1:4-dihydroxy-2:3-naphthalene dicarboxylic acid diphosphate (Compound XI) on chick fibroblast cultures. Concentration 6×10^{-6}M. Fixed after 24 hours.

FIG. 6.—Enlarged cell. Metaphase with irregular distribution of chromosomes, partial chromosome clumping and some chromosomes showing regions of defective stainability. × 1200.

FIG. 7.—Metaphase with gross clumping of chromosomes. × 1200.

FIG. 8.—Degenerate cell and débris of many breaking-down cells. × 1200.

FIG. 9.—Enlarged cell, probably anaphase with irregular tripolar spindle, anaphase bridge and chromosome fragmentation. × 875.

FIG. 10.—Effect of dicalcium salt of 2:3:6-trimethyl-hydroquinone diphosphate (Compound XIV) on chick fibroblast cultures. Concentration 6×10^{-6}M. Fixed after 24 hours. Enlarged cell. Tripolar metaphase. × 1200.

FIG. 11–18.—Effects of tetra-sodium 2:3-dimethyl-1:4-naphthohydroquinone diphosphate (Compound XXVIII) on chick fibroblast cultures. Fixed after 24 hours.

FIG. 11.—Control. × 185.

FIG. 12.—At 24 hours after application of compound XXVIII in concentration 6×10^{-6}M. Metaphase accumulation. × 185.

FIG. 13–18.—At 24 hours after application in concentration 4×10^{-6}M. × 1175.

FIG. 13.—Anaphase bridges, chromosome fragments and cytoplasmic vacuolation outside the spindle area.

FIG. 14.—Anaphase bridges.

FIG. 15.—Two cells showing metaphase arrest with a clearly defined spindle, some irregularity of chromosome arrangement and cytoplasmic vacuolation outside the spindle area.

FIG. 16.—Three cells in metaphase showing gross clumping of chromosomes.

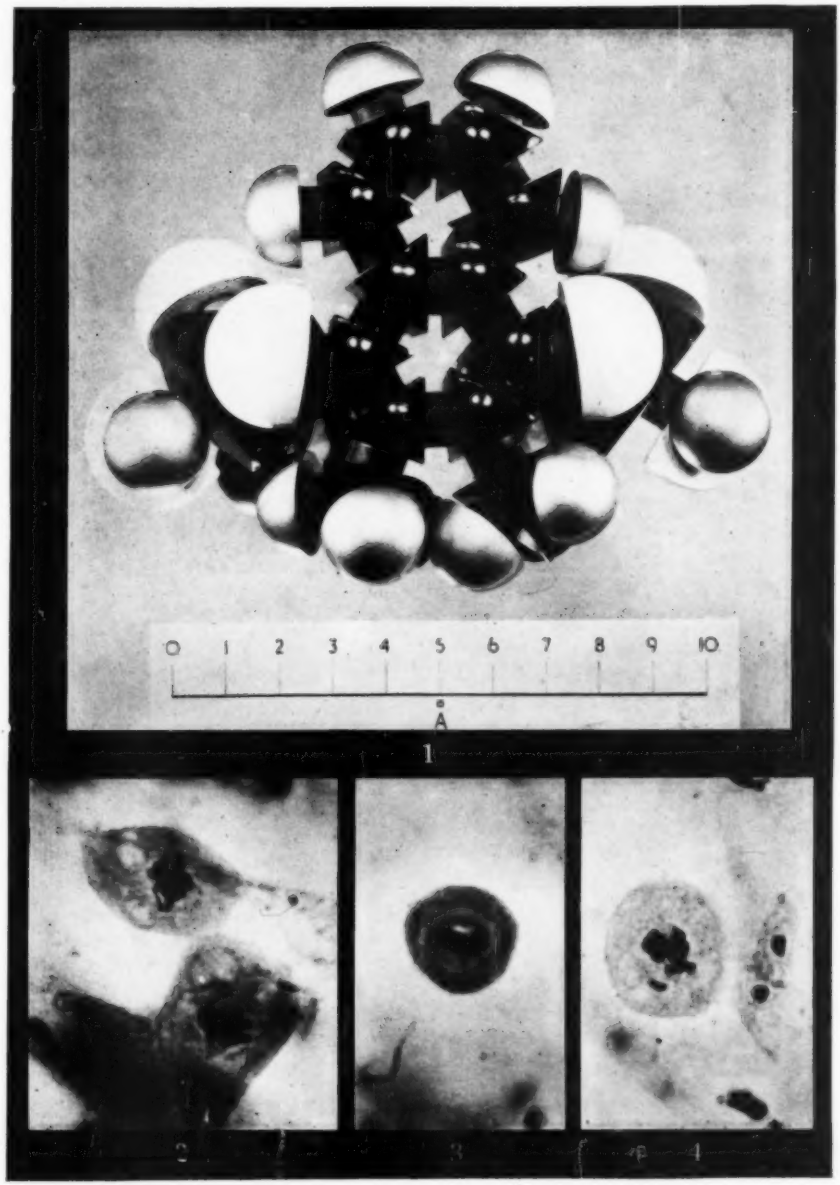
FIG. 17.—Distorted metaphase with incomplete spindle formation and cytoplasmic vacuolation.

FIG. 18.—Enlarged cell; prometaphase with irregular arrangement of chromosomes, and chromosomes showing regions of defective staining.

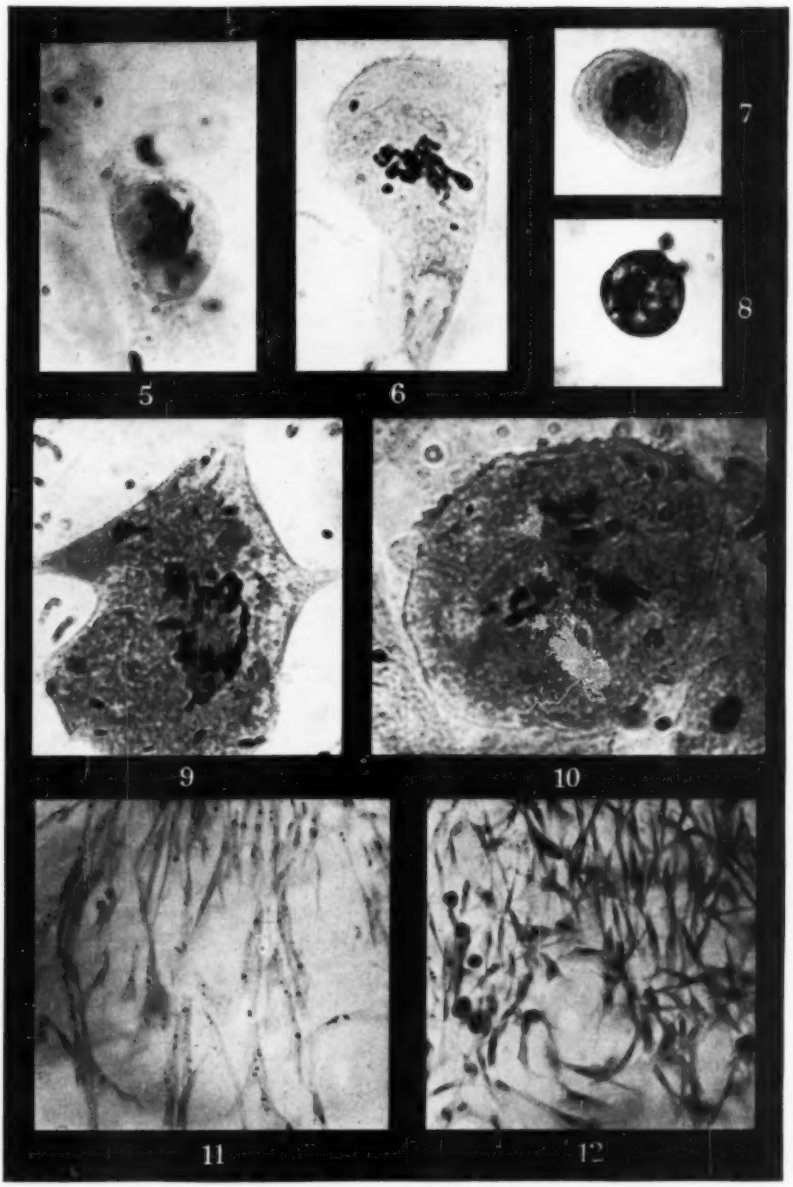
FIG. 19, 20.—Effect of Vitamin K_1 (Compound XXIX) on chick fibroblast cultures. Concentration 5×10^{-6}M. in Cellosolve and gum Ghatti. Fixed after 24 hours. × 1175.

FIG. 19.—Metaphase with partial chromosome clumping.

FIG. 20.—Anaphase with spindle abnormality and chromosome bridge and fragments.



Mitchell and Simon-Reuss.



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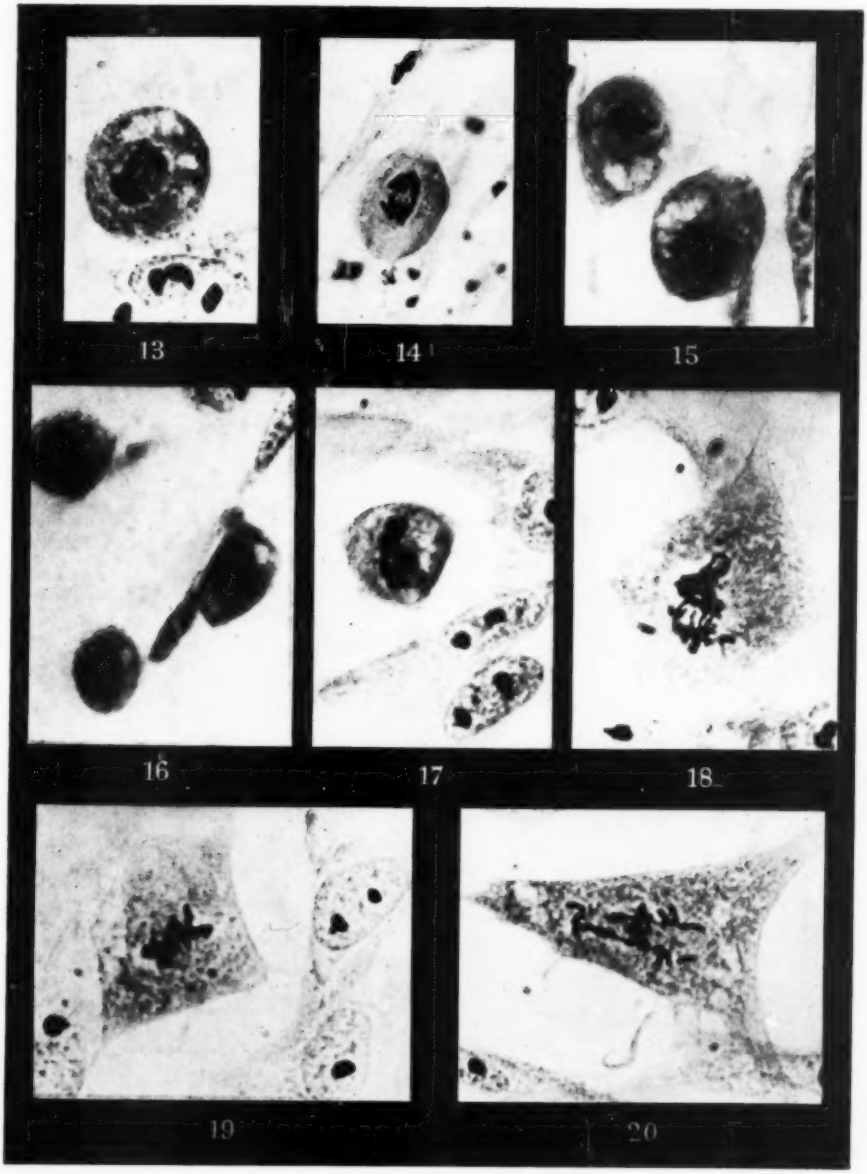
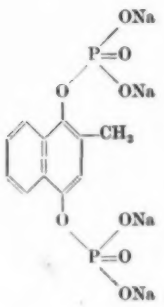


Mitchell and Simon-Reuss.

TABLE II.



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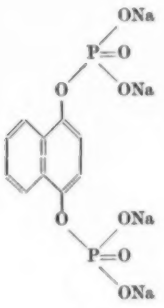

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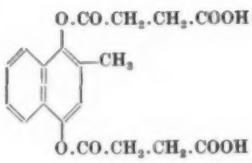

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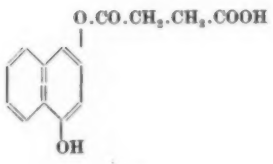

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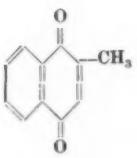

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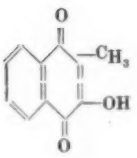

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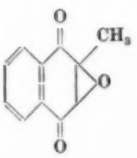

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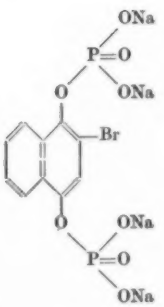

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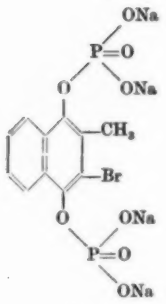

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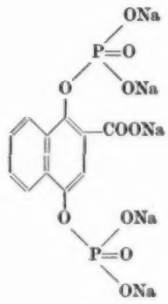

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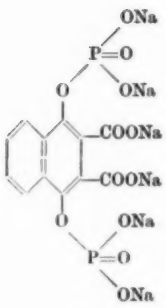

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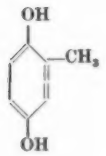

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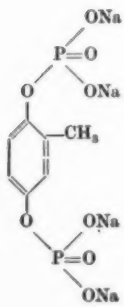

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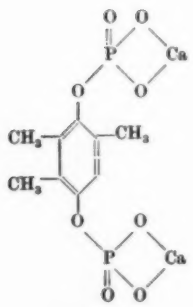

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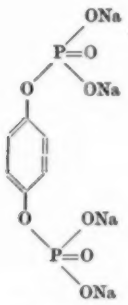

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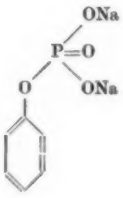

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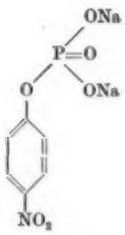

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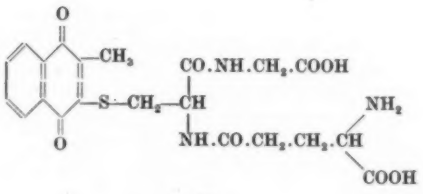

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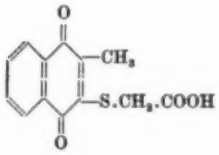

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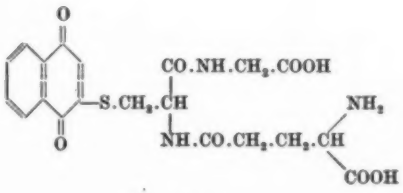

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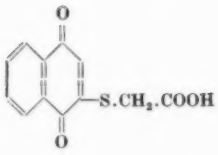

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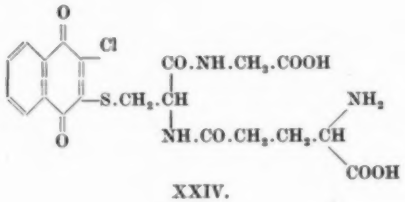

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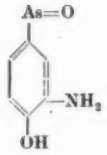

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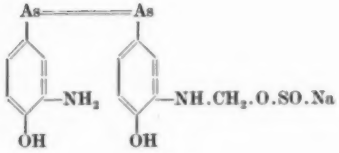

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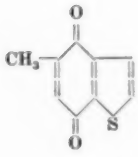

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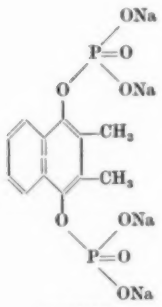

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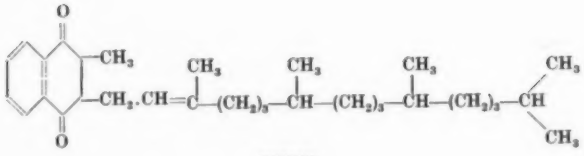

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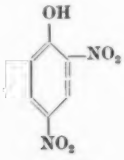

XXX.

Among the simpler compounds toluhydroquinone diphosphate (XIII) is more active as a mitotic inhibitor than toluhydroquinone (XII) by a factor of about 5. On the basis of the concentration required to produce 50 per cent mitotic inhibition, hydroquinone diphosphate (XVI) is about twice as active as hydroquinone (XV), but the increase of mitotic inhibition with concentration is much more rapid in the case of the former than with the latter. Somewhat similarly, for 50 per cent mitotic inhibition, phenyl phosphate (XVIII) is more active than phenol (XVII) by a factor of about 20, but the increase of mitotic inhibition with concentration is much less rapid in the case of phenyl phosphate than for phenol.

The only exception so far to the finding of increased antimitotic activity on phosphorylation is in the case of resorcinol. Dr. Friedmann and Mrs. Simon-Reuss found that 50 per cent mitotic inhibition is produced by resorcinol at 6×10^{-8}M concentration but by resorcinol diphosphate at about 5×10^{-7}M. At these concentrations both compounds showed few abnormal mitoses.

The usual increase in antimitotic activity on phosphorylation is not associated with any change in character of the cytological effects. The frequency of cytological abnormalities appears to be of the same order for the phosphorylated and non-phosphorylated compound at concentrations which produce the same mitotic inhibition.

(2) *Methyl group.*

The methyl group plays an important part in modifying the biological action, especially from the point of view of practical applications. Moreover, among methyl derivatives, the limitations of the use of mitotic inhibition as a sorting test are emphasised.

In the chick fibroblast cultures, Compound II is more active than Compound I as a mitotic inhibitor by a factor of about 1000, and it also shows potentiation of mitotic inhibition in combination with X-rays. However, Compound II appears to be ineffective as a radiosensitiser for the Jensen rat sarcoma, and did not produce a focal reaction in the tumour after intravenous injection in a few cases in man. It may be mentioned that its use in man in high doses may be undesirable because of the possibility of induction of cataract, analogous to the experimental naphthalene cataract in rabbits (Bourne, 1937).

It is of interest to compare the methyl free Compound II, tetra-sodium 1:4-naphthohydroquinone diphosphate, with the 2-methyl derivative, Compound I, and with the 2:3-dimethyl derivative, Compound XXVIII. Compound I produces remarkably few mitotic abnormalities and no change in mitotic phase distribution even at 10 times the concentration corresponding to 50 per cent mitotic inhibition. No other compound examined showed this behaviour. Compound II showed an increase in the number of metaphases by a factor of about 2·5 on increasing the concentration for 50 per cent mitotic inhibition by a factor of 10.

It has been shown in the preceding paper (Mitchell and Simon-Reuss, 1952) that Compound I is somewhat unstable in aqueous solution in the absence of oxygen at 39° C. The increase in stability in Compounds II, I, and XXVIII on methylation is correlated with the decrease in the oxidation-reduction potential of the corresponding dephosphorylated compounds, which has the values at pH7 of +71, −5, and −73 millivolts respectively (Trenner and Bacher, 1941).

Steric interference of the free rotation of the adjacent methyl and phosphate groups may be of importance especially in Compound XXVIII (Fig. 1), where it probably tends to fix the distance between the 1- and 4-phosphate groups.

In Compound XXVIII the distance between the centres of the oxygen atoms in the two most distant ionisable phosphoric ester hydroxyl groups when these are in what may be termed a " cis " position lies between 9·5 and 10·5Å. It is of interest that this distance is close to the value of 10·2Å found for the repeating distance along some polypeptide chains and comprising 6 amino acid residues (Bragg, Kendrew and Perutz, 1950). One may speculate and suggest the possibility of combination of the phosphates with arginine side chains of histones in the chromosome structure.

Biological action of 2:3-*dimethyl*-1:4-*naphthohydroquinone diphosphate, tetrasodium salt* (*Compound XXVIII*).—The biological action of Compound XXVIII differs considerably from that of all the other compounds examined (Fig. 11 to 18). It blocks the entry of cells into mitosis, and in concentrations which inhibit mitosis has acquired strong activities for the production of—

(1) arrest of mitosis in metaphase together with spindle abnormalities similar to those produced by colchicine, and
(2) chromosome fragmentation and anaphase bridges.

For 50 per cent mitotic inhibition it is only about half as active as Compound I. Even in concentration 2×10^{-6}M (though not at 5×10^{-7}M), which produces no detectable mitotic inhibition, there is metaphase accumulation by about 50 per cent above the fraction of metaphases in the controls, together with spindle abnormalities.

As an example of the frequency of relevant chromosome aberrations, it will be seen from Table III that after application in concentration 4×10^{-6}M for 24 hours, out of 534 cells in mitosis, there were 74 cells in metaphase showing chromosome fragmentation and 17 anaphase bridges. It appears that with Compound XXVIII there is more rejoining of chromosome breaks than with any of the other compounds examined. Moreover, Compound XXVIII only starts to show effects on resting cells in high concentrations associated with almost complete mitotic inhibition ; this behaviour suggests low toxicity.

Detailed studies of the combination of the effects of Compound XXVIII and X-radiation on the chick fibroblast cultures have been made by the summation method. The results of one experiment are given in Table III.

It is concluded that under the experimental conditions, the combination of the action of Compound XXVIII and of X-radiation shows potentiation of mitotic inhibition and that the mechanism of action of the two agents is different.

(3) *Carboxyl group.*

The compound most closely resembling Compound XXVIII in biological action is Compound XI, the hexasodium salt of 1:4-dihydroxy-2:3-naphthalene dicarboxylic acid diphosphate. Replacement of the methyl groups in the 2 and 3 positions in Compound XXVIII by carboxyl groups slightly reduces the activity as measured by mitotic inhibition and considerably reduces the metaphase effects, so that at 50 per cent mitotic inhibition there is metaphase accumulation by about 25 per cent and spindle abnormalities are much less frequent. Compound XI produces anaphase bridges like Compound XXVIII, though less

TABLE III.—*Combination of Effects of Compound XXVIII and X-Radiation in Chick Fibroblast Cultures.*

Treatment.	Time of fixation after treatment (hours).	Number of cultures.	Number of mitoses in individual cultures.						Total number of cells in mitosis.	Per cent mitotic inhibition.
a. Control	24	6	114	112	115	108	121	117	687	—
b. 4×10^{-6}M Compound XXVIII	24	6	61	81	124	51	67	150	534	22·3
c. 300 r X-radiation	6	6	71	63	65	65	66	61	391	43·1
d. 2×10^{-6}M Compound XXVIII followed after 18 hours by 150 r X-radiation	24	6	4	8	7	0	12	1	32	95·3

The mean of the effects of 4×10^{-6}M compound (*b*) and 300 r X-radiation (*c*) is compared with the effect of the combination (*d*) of half quantities of the two agents. The mean mitotic inhibition in (*b*) and (*c*) is 32·7 per cent while the mitotic inhibition in (*d*) is 95·3 per cent. Comparing the numbers of mitoses in the individual cultures by the use of the means of paired cultures in (*b*) and (*c*) and the observed numbers in (*d*), for 10 degrees of freedom, Student's t is 9·40 and P is much less than ·001. Hence the mitotic inhibition in (*d*) is significantly higher than the mean mitotic inhibition in (*b*) and (*c*), and potentiation is confirmed.

Chromosome fragmentation was found in 74 cells in mitosis in (*b*), 19 in (*c*) and 3 in (*d*), and anaphase bridges respectively 17, 0, and 1. The mitotic inhibition in (*d*) is so large that it is only possible to conclude that there is no gross departure from the expected proportion of cells with chromosome fragmentation among the surviving mitoses.

frequently, but appears to be much more toxic, as shown by the occurrence of large numbers of exploded and degenerating mitotic cells and rounded and vacuolated resting cells at a concentration producing about 60 per cent mitotic inhibition (Fig. 6 to 9).

In Compound I replacement of the 2-methyl group by carboxyl to give Compound X slightly reduces the activity for mitotic inhibition and considerably increases the toxic action on resting cells. However, Compound X like Compound I produces few abnormal mitoses and practically no anaphase bridges.

These results suggest the possibility of intra-cellular oxidation of the methyl group in Compound I and of one of the two methyl groups in Compound XXVIII. Probably penetration into the cell is reduced by replacement of the 2-methyl group by carboxyl.

(4) *Other groups.*

(i) *Bromine.*—The introduction into the molecule of halogens, especially bromine and iodine, has been studied from the point of view of preparing radioactive labelled analogues which may concentrate in tumours. Bromine appears to present less difficulty than iodine.

Bromine and methyl have almost the same van der Waals radii, but opposite inductive effects and very different chemical properties. Replacement of the 2-methyl group in Compound I by bromine to give Compound VIII results in little change in the concentration required to give 50 per cent mitotic inhibition, but produces metaphase accumulation at higher concentrations and greatly increases the toxicity. The Compound IX, tetra-sodium 2-methyl-3-bromo-1:4-naphthohydroquinone diphosphate, produces 50 per cent mitotic inhibition in almost exactly the same concentration as the dimethyl Compound XXVIII, but metaphase accumulation only at the lowest concentration examined, 1×10^{-5}M, and practically no anaphase bridges (Fig. 2 to 5). Although it produces many more abnormal mitoses, Compound IX is closer in its biological action to Compound I than to Compound XXVIII.

The replacement of 2-methyl in the addition product, S-(2-methyl-1:4-naphthoquinonyl-3-) glutathione (XX), by 2-chloro- gives the Compound XXIV, which has lost its biological activity. This may be due to failure of intra-cellular oxidation of the methyl group.

(ii) *Hydroxyl group.*—The introduction into 2-methyl-1:4-naphthoquinone of a hydroxyl group in the 3-position to give phthiocol (VI) is associated with mitotic inhibition almost exactly equal to that of Compound I, which is the diphosphate. Compound VI produces clumped metaphases with some chromosome fragmentation and many undivided telophases, but practically no anaphase bridges. Metaphase accumulation appears at a concentration which produces about 70 per cent mitotic inhibition ; this is associated with negligible effect on resting cells.

The weak metaphase arrest with Compound VI is perhaps associated with the electron-repelling inductive effect of the hydroxyl group. It is interesting to note that its oxidation-reduction potential at pH7 has the low value of −113 millivolts (Trenner and Bacher, 1941).

Iso-naphthazarin (2:3-dihydroxy-1:4-naphthoquinone) has been studied by Dr. Friedmann and Mrs. Simon-Reuss. It produces an increase in the number

of mitoses, rising to 50 per cent in 1×10^{-5}M concentration above the number in the controls. Phase distribution studies confirmed by the recording film technique show that the increase is due to metaphase accumulation. It appears that this 2:3-dihydroxy-compound has lost its activity for blocking the entry of cells into mitosis but has acquired strong activity for the arrest of mitosis in metaphase.

(iii) *Oxide.*—Compound VII, 2-methyl-1:4-naphthoquinone-2:3-oxide, is inactive. This compound is of interest, because it is probably responsible for the yellow fluorescence with Wood's light which appears in the growing parts of the Walker Rat Carcinoma 256 and in some other tissues at least 6 hours after intramuscular or intravenous injection of Compound I (Mitchell, 1951).

(iv) *Nitro group.*—The introduction of the p-nitro group into Compound XVIII to give Compound XIX is associated with reduction of activity for mitotic inhibition by a factor of 10 and a great increase in toxicity. It is well known that p-nitro-phenol is the most toxic of the mononitrophenols; it is considered probable that its toxicity depends on the rate of reduction (Williams, 1947, p. 136).

It is of interest to study dinitrophenol (Compound XXX), since in concentrations in the region of 10^{-5}M it has been shown to produce inhibition of phosphorylation without affecting or with slight stimulation of oxidation in rabbit kidney homogenate (Loomis and Lipmann, 1948), and reduction of the rate of cleavage and uptake of P^{32} in the developing sea urchin embryo (Villee, Lowens, Gordon, Leonard and Rich, 1949); see also review by McElroy (1947) and Clowes (1951).

Dinitrophenol rather surprisingly produces 50 per cent mitotic inhibition in a concentration lower by a factor of 2 than phenol (XVII). Its toxic effects only become severe at a concentration higher than the MI50 by a factor greater than 2, so that it is rather less toxic than might have been anticipated. The increasing metaphase accumulation at the higher concentrations observed with dinitrophenol is not found with phenol and suggests some difference in the mechanism of action.

These results are not inconsistent with the view that inhibition of the entry of cells into mitosis depends upon the prevention of some synthetic processes by inhibition of phosphorylation without interference with carbohydrate breakdown. It is possible that metaphase arrest involves a similar but less sensitive mechanism. However, much more biochemical evidence is required.

(v) *Phytyl group.*—Vitamin K_1, 2-methyl-3-phytyl-1:4-naphthoquinone (XXIX), in concentration 5×10^{-6}M, produces no mitotic inhibition and no change in mitotic phase distribution, but nevertheless results in chromosome fragmentation and some anaphase bridges (Fig. 19, 20). At higher concentrations there is a toxic effect on resting cells.

These findings confirm the independence of the processes of mitotic inhibition and of chromosome breakage and reunion.

(5) *Ring structure.*

Considerable alteration of the ring structure may be tolerated without gross change in biological activity, but is usually associated with increased production of abnormal mitoses.

5-Methyl-4:7-thionaphthene quinone (XXVII) is active when tested in solution in Cellosolve and gum Ghatti, and produces 50 per cent mitotic inhibition in approximately 5×10^{-6}M concentration. At this concentration it produces some abnormal mitoses, mainly clumped metaphases, and at higher concentrations there is metaphase accumulation. There are practically no anaphase bridges and there is little visible effect on resting cells, which suggests low toxicity.

The dicalcium salt of 2:3:6-trimethyl-hydroquinone diphosphate (XIV) is only a little less active as a mitotic inhibitor than Compound I but produces many abnormal mitoses (Fig. 10). In a concentration which produces 70 per cent mitotic inhibition there are many rounded-up and disconnected resting cells.

The tetra-sodium salt of toluhydroquinone diphosphate (XIII) is less active as a mitotic inhibitor than Compound (XIV) by a factor of 2, but produces metaphase accumulation and abnormal mitoses, mainly clumped metaphases, chromosome fragmentation and multipolar cells, at all concentrations showing mitotic inhibition.

The tetra-sodium salt of hydroquinone diphosphate (XVI) is a very active compound, and produces 50 per cent mitotic inhibition at almost exactly the same concentration, 3×10^{-9}M, as does Compound II. In this region of concentration it also produces few abnormal mitoses. Thus the action of the hydroquinone diphosphate (XVI) resembles that of the naphthohydroquinone diphosphate (II), to which it may be regarded as more closely related than to Compound I.

In the case of phenyl phosphate (XVIII), the observed slow increase in mitotic inhibition with concentration suggests the possibility of its conversion in the cell rather inefficiently to hydroquinone.

(6) *Interaction with sulphydryl compounds.*

It was thought at first that interaction with –SH groups was likely to be an important factor in mitotic inhibition (Brachet, 1947, p. 184) and radiosensitisation (Barron and Dickman, 1949, Patt, Smith, Tyree and Straube, 1950). Evidence was soon obtained for a general parallelism between the production by different compounds of mitotic inhibition in the chick fibroblast cultures, and the formation *in vitro* by the parent naphthoquinones of addition products with sulphydryl compounds. For example, 1:4-naphthoquinone and 2-methyl-1:4-naphthoquinone readily add –SH compounds. Similarly, with simpler compounds it was found that maleic acid, which produces 50 per cent mitotic inhibition at about 5×10^{-7}M concentration, adds –SH compounds while the *trans* isomer, fumaric acid and the methyl derivatives citraconic and mesaconic acids show no –SH uptake and do not produce mitotic inhibition. However, although not a mitotic inhibitor, fumaric acid produces many mitotic abnormalities, including chromosome fragmentation, multipolar cells and exploded cells as well as clumped metaphases. There was no change in mitotic phase distribution.

Further studies showed many exceptions. While Compounds XXI, XXII, XXIII and XXIV, as expected, produced no mitotic inhibition and no cytological abnormalities, the important addition product, S-(2-methyl-1:4-naphthoquinonyl-3-) glutathione, is more active in low concentrations as a mitotic inhibitor than

Compound I by a factor of 2, and also produces mitotic abnormalities with slight metaphase accumulation at the lower concentrations. Its mitosis-concentration relation is anomalous, the maximum mitotic inhibition reached being about 55 per cent.

Another line of approach was to study the arsenicals. It is generally accepted that the arsenoxides combine with –SH groups. However, mapharside (XXV) produces no mitotic inhibition and no change in mitotic phase distribution in concentrations up to and including 5×10^{-6}M, although it is rather toxic and affects the resting cells. Neoarsphenamine (XXVI) is more toxic but produces no mitotic inhibition and no change in mitotic phase distribution, though there is some chromosome clumping; it is to be noted that in these, as in the other experiments, the neoarsphenamine (XXVI) was applied to the cultures for 24 hours.

From the point of view of possible applications, the most important exceptions are the 2:3-disubstituted naphthohydroquinone diphosphates, Compounds IX, XI and especially XXVIII. It is well known that compounds of this type are completely inert to sulphydryl compounds (Fieser and Fieser, 1944, p. 739). Further, experiments to test this point, kindly made by Dr. K. Bailey have shown that Compound XXVIII does not take up –SH groups in 3 hours at pH7 and room temperature, nor does 2:3-dimethyl-1:4-naphthoquinone.

It must be concluded that mitotic inhibition, mitotic abnormalities and radiosensitisation can be produced by compounds which do not interact *in vitro* with sulphydryl groups.

(7) *Absence of correlation with Vitamin K activity.*

The following values for the approximate Vitamin K activity in Dam-Glavind units per mg. were given by Dam, Glavind and Karrer (1940) and Dam (1942): Compound I, $3{\cdot}75 \times 10^4$; 1:4-naphthoquinone, 50; Compound III, $1{\cdot}5 \times 10^4$; Compound V, $2{\cdot}5 \times 10^4$; phthiocol, Compound VI, 50–100; Compound VIII is stated to be less active than the quinone; Compound XXVIII, "O," and Vitamin K_1, Compound XXIX, $1{\cdot}2 \times 10^4$.

Comparison with Table I shows that for the compounds studied there is no correlation between the Vitamin K activity and the activity for the production of mitotic inhibition and cytological abnormalities in the chick fibroblasts in culture, under the present experimental conditions.

DISCUSSION.

These experiments show that the main biological effects studied, namely, inhibition of the entry of cells into mitosis, chromosome breakage and radiosensitisation of mitotic inhibition, can be produced by compounds which do not interact *in vitro* with sulphydryl groups. Moreover, they are not produced by some compounds which interact characteristically with –SH groups. In some cases there is the possibility of metabolic transformation of the compounds by the living cells to give intermediate products which interact with –SH groups. However, it must be concluded that these biological effects are not directly dependent upon interactions with substances containing sulphydryl groups, although it is likely that such interactions play some part.

In this series of compounds the activity for mitotic inhibition is almost in-

variably increased by phosphorylation, often by a factor of 5 or more. These results and those with dinitrophenol suggest that blockage of the entry of cells into mitosis depends upon inhibition of synthetic processes involving phosphorylation. It is interesting that from the non-protein moiety of a phosphorylase has been isolated a yellow pigment tentatively identified as 2-methyl-1:4-naphthoquinone (Buell, 1952). It seems unlikely that at the concentrations used there is interference with glycolysis (Gemmill, 1949).

The comparison of the cytological effects of different compounds of closely related chemical constitution and also of ionising radiations makes it possible to separate some of the main types of mechanism of action of these agents on proliferating cells as follows :

(*a*) Blockage of the entry of cells into mitosis, without specification of the exact time of the action and presumably including pre-prophase and antephase inhibition. This is the characteristic action of Compound I, and in this case is associated with relatively few chromosomal abnormalities. Other less clearly defined examples are Compounds X and XV.

(*b*) Metaphase arrest and disturbances of the spindle mechanism apparently similar to the effects of colchicine. Examples of this type of action in association with (*a*) are Compounds II, VI, VIII, XI, XXVII and XXX at the higher concentrations, Compound IX at low concentrations and Compound XXVIII over a wide range of concentrations. Iso-naphthazarin produces metaphase arrest with little or no inhibition of the entry of cells into mitosis.

(*c*) Chromosome, including chromatid, breakage with varying degrees of rejoinability of the broken ends. Chromosome breakage without anaphase bridge formation is found with Compounds VIII, IX, XII, XIII, XIV and XXX. Chromosome breakage with anaphase bridge formation is observed characteristically with X-radiation and with Compound XXVIII ; considerably fewer anaphase bridges are observed with Compound XI. In all these instances chromosome breakage is associated with blockage of the entry of cells into mitosis. With Vitamin K_1 (Compound XXIX) there is no mitotic inhibition, but chromosome breakage and anaphase bridges are observed together with some spindle abnormalities.

In association with chromosome breakage, chromosomes showing regions of impaired stainability often with irregular and beaded structure are found with Compounds I (in low frequency), IX, XI and XXVIII ; similar changes can occur but are infrequent with X and γ rays. These appearances suggest the possibility of two mechanisms of chromosome breakage, namely, "direct action" breaks similar to those produced by the passage of an ionising particle through or near a chromosome thread, and " metabolic " breaks due to impaired synthesis of the molecular structure of the chromosome.

It seems possible to assess very roughly the toxicity of a compound by observing the effects on the resting cells in the chick fibroblast cultures. Characteristic appearances are that the resting cells become disconnected and rounded up, with retraction of the cell processes. Changes of this type are seen with Compounds VIII, XI, XIV, XIX, XXV, XXVI and XXX in concentration 1×10^{-5}M or less. Our own animal experiments confirmed the high toxicity of Compound VIII.

The evidence obtained in these cytological studies suggests that the tetrasodium salt of 2:3-dimethyl-1:4-naphthohydroquinone diphosphate, Compound

XXVIII, is the most suitable compound for further investigation as a possible therapeutic radiosensitiser. It has been confirmed experimentally in the chick fibroblast cultures (Table III) that the combination of the effects of Compound XXVIII and of small doses of X-radiation shows potentiation of mitotic inhibition. The mitotic inhibition observed with the combined agents was so great that it was not possible to make satisfactory quantitative studies of chromosome fragmentation. It is of interest that in animal experiments Compound XXVIII is of low toxicity, and accumulates in the actively growing parts of the Walker Rat Carcinoma 256 and some other tissues after intramuscular injection, as shown by the fluorescence method used previously for Compound I (Mitchell, 1950, 1951). Clinical trials have been started.

Another compound which appears to merit further examination is the tetra-sodium salt of 2-methyl-3 bromo-1:4-naphthohydroquinone diphosphate, Compound IX.

SUMMARY.

1. The cytological effects of 29 selected compounds related to tetra-sodium 2-methyl-1:4-naphthohydroquinone diphosphate have been examined from the points of view of the relation of the biological actions to the chemical constitution and the selection of new compounds as possible therapeutic radiosensitisers.

2. The comparison of the cytological effects of the closely related compounds and also of ionising radiations makes it possible to separate three main types of mechanism of action on proliferating cells :

(a) blockage of the entry of cells into mitosis,

(b) metaphase arrest and spindle abnormalities apparently similar to the effects of colchicine, and

(c) chromosome breakage and its effects.

The appearances suggest the possibility of two types of chromosome breaks, which may be termed " direct action " breaks and " metabolic " breaks.

3. These biological effects are not directly dependent upon interactions with substances containing sulphydryl groups, although it is likely that this type of interaction plays some part.

4. It is suggested that inhibition of the entry of cells into mitosis depends on blockage of cellular synthetic processes involving phosphorylation.

5. These experiments suggest that the tetra-sodium salt of 2:3-dimethyl-1:4-naphthohydroquinone diphosphate, Compound XXVIII, is the most suitable compound for further investigation as a possible therapeutic radiosensitiser.

In addition to the help acknowledged in the text, Dr. E. J. Friedmann has given permission to refer to some of his unpublished work, especially that on phenols. We are grateful to Dr. Malcolm Dixon for suggesting the desirability of examining the dimethyl derivative, Compound XXVIII. We wish to thank Miss D. Brown and Mr. E. W. Mitchell for skilled technical assistance.

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